Vol. XL No. 11

November, 1951

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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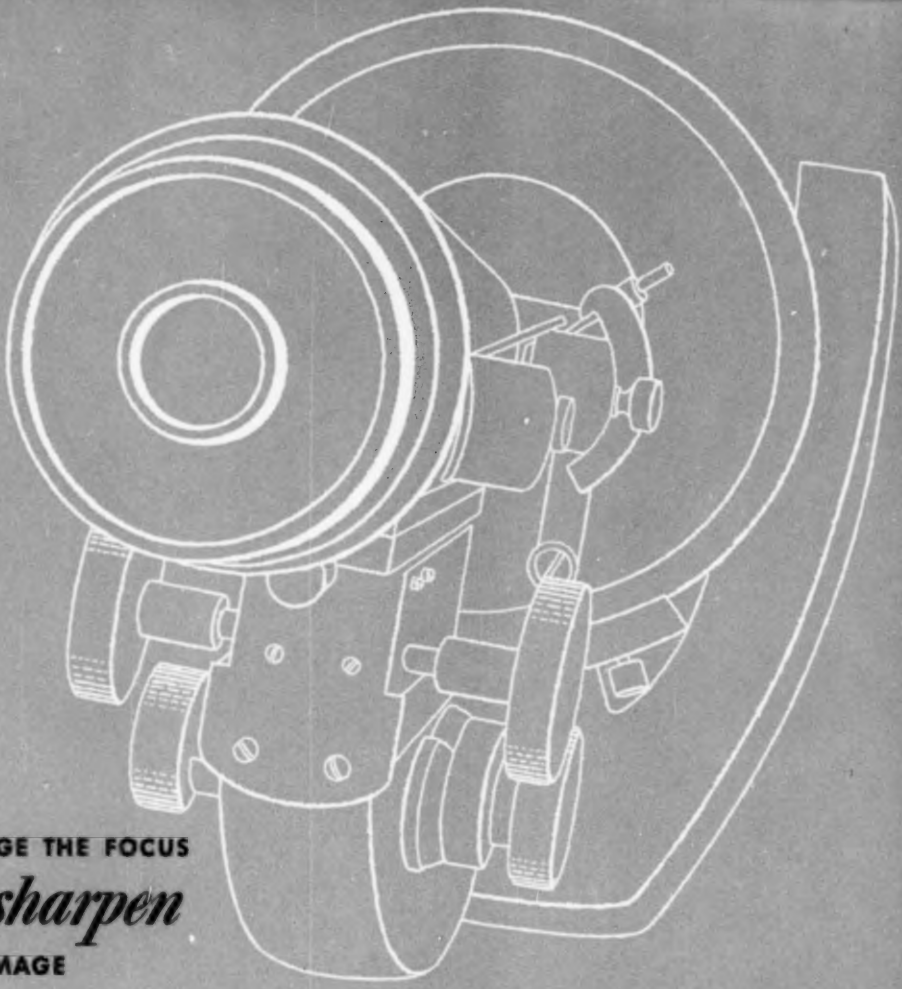

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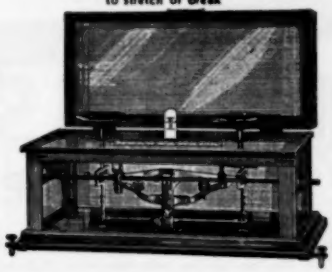

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VOLUME XL — *NOVEMBER, 1951* — NUMBER 11 — CONSECUTIVE NO. 22

Terramycin in Serum and Urine After Ingestion of Terramycin Base*,†

By JEAN D. CARLILE, ALICE C. KESTER, C. BURR McDONALD, and CARL F. CLANCY

After ingestion of a single 2.0-Gm. dose of terramycin base the drug was detected in serum as soon as one to two hours and in approximately the same concentrations as that found in subjects who received equivalent doses of terramycin hydrochloride. When the base was given 0.5 Gm. every six hours it appeared in the serum of some subjects by the eighth hour and all subjects had demonstrable levels on the twenty-fourth hour. Terramycin appears in the urine within an hour after ingestion of 2.0 Gm. of the base compound and small amounts were still detectable in specimens voided on the twenty-fourth hour. Lower drug levels were attained in urine from the base than after ingestion of an equal amount of terramycin hydrochloride.

STUDIES on the concentrations of terramycin in serum and urine after ingestion of terramycin hydrochloride have been reported by Welch, Hendricks, Price, and Randall (1), by Herrell, Heilman, Wellman, and Bartholomew (2), and by Schoenbach, Bryer, and Long (3). The hydrochloride compound is soluble in distilled water or physiological saline, but the parent compound, terramycin base, is quite insoluble within the physiological *p*H range. Since the drug is usually administered orally, it is of interest to compare absorption and excretion of terramycin when administered orally as the hydrochloride and as the base, especially since it is recognized that under certain conditions free hydrochloric acid is present in the stomach. Accordingly, experiments were conducted to study blood and urine levels of terramycin activity after ingestion of single doses and also after divided doses of the two forms of the antibiotic.

SUBJECTS

Twenty human subjects were selected, all of whom were presumably healthy males twenty-five to thirty years of age, living for the most part in the same environment. Although no studies on free hydrochloric acid in the stomachs of the subjects were made for this report, the men are in an age group in which the chance occurrence of achlorhydria is unlikely. The experiments were started at 8 a. m., subjects being in the fasting state, but thereafter no restrictions were placed upon food or fluid intake.

ASSAYS

Assays of both serum and urine specimens were carried out by the twofold serial dilution method using Pen-assay broth, the volumes being adjusted so that 0.5-ml. amounts of inoculum (twenty-four-hour broth culture of *Staphylococcus aureus* 209P diluted 1:1,000) were added to equal volumes of the

* Received June 18, 1951, from the Ayer Clinical Laboratory of the Pennsylvania Hospital, Philadelphia, Pa.

† This study was supported by a grant from Chas. Pfizer and Co., Brooklyn, N. Y. The terramycin base and terramycin hydrochloride were kindly supplied by Dr. Elliott R. Weyer of the same company.

TABLE I.—RESULTS OF TERRAMYCIN BASE STUDIES

Expt. No.	Spec. Assayed	Average Drug Recd.	Dosage	Levels Attained, μg. Terramycin per Ml. Serum or Urine — Hours 0	1	2	4	8	24
1	Serum	HCl	Single dose 2.0 Gm. at 8 a. m.	0	0	1.9	4.8	1.2	0
		Base		0	0.9	1.8	2.4	1.9	0
2	Serum	HCl	Divided doses 0.5 Gm. Q6H	0	0	0	0	0	1.6
		Base		0	0	0	0	0.6	1.1
3	Urine	HCl	Single dose 2.0 Gm. at 8 a. m.	0	45	46	778	471	328
		Base		0	37	171	237	309	79

dilutions of serum and urine. The test organism, *St. aureus* 209P, was found to be consistently sensitive to amounts of from 0.1 to 0.2 μg. of terramycin per ml. when tested against known concentrations of the drug. The known standards were prepared from terramycin hydrochloride (*p*H 2.2) hydrated to 5,000 μg./ml. (Pfizer terramycin testing kit). Serums and urines were collected asceptically and stored at −20° until it was convenient to do the assays. In this way all the assays were completed within three to four days and thus the results are probably more comparable.

RESULTS AND DISCUSSION

Experiment 1.—Fifteen subjects were given 2 Gm. of terramycin base orally at 8 a. m. and bled at intervals, as noted in Table I, during the course of the day. Five control subjects received the same amount of terramycin hydrochloride and their bloods were similarly drawn for terramycin assay. As may be seen from Table I, enough of the terramycin base had been absorbed from the alimentary tract to yield an average level of 0.9 μg./ml. of serum at the end of the first hour. It should be noted, however, that not all of the subjects showed a measurable level of drug at this time. All subjects had demonstrable terramycin present two hours after receiving terramycin base, the average level being 1.8 μg./ml. of serum. The highest level occurred between the second and eighth hours, the fourth hour average being 2.4 μg./ml. Although drug was still present in the serum after eight hours, none was present after twenty-four hours. The levels on the control subjects who received the hydrochloride were about the same as those on the subjects who received the base, except that the fourth hour serum levels from the hydrochloride were higher. These terramycin levels attained after ingestion of terramycin base are comparable to the results attained by Mason, Kice, and Caffery (4) after ingestion of 2 Gm. of the base compound. It should be noted that the average levels attained in the subjects who received terramycin hydrochloride were similar to those reported by Welch, *et al.* (1), Herrell, *et al.* (2), and Schoenbach, *et al.* (3).

Experiment 2.—To observe the appearance of terramycin activity in the serum of subjects on divided doses of terramycin, the subjects were grouped as before, 15 of whom received the base and five the hydrochloride, 0.5 Gm. every six hours. By the eighth hour some of those receiving the base showed demonstrable levels of drug, the average being 0.6 μg./ml. and by the twenty-fourth hour both groups showed approximately the same amounts of drug in their serum (Table I).

Experiment 3.—The assays of terramycin in urine after ingestion of a single dose of 2.0 Gm. of terramycin base and hydrochloride are also summarized in Table I. In this particular instance all subjects were excreting the drug by the end of the first hour, at which time in Experiment 1, several subjects who received the base had demonstrable serum levels, while others did not. Furthermore, both groups were still excreting small amounts of terramycin twenty-four hours after receiving the drug, at which time studies on the serum had failed to show any terramycin. The levels attained in subjects who received the base were in general lower than in those who received the hydrochloride. An exception was at the end of the second hour when the average μg./ml. was 171 for those receiving the base compound, while the control group at that time was 46 μg./ml. However, the average of 171 was heavily weighted by the levels of two subjects, each of whom showed levels of 819 μg./ml. at the end of the second hour.

Thus it is apparent that after ingestion of the base compound, terramycin is not concentrated in the urine to the same degree as following ingestion of equivalent weights of the hydrochloride compound. Further studies might elucidate whether or not this difference represents a failure to absorb the base or a difference in its metabolism by the body. From the studies on the serum levels in this report and those by Mason, *et al.* (4), and by Musselman (5), it appears that the terramycin is absorbed from the alimentary tract at about the same rate regardless of whether the base or the more soluble hydrochloride is ingested.

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(1) Welch, H., Hendricks, F. D., Price, C. W., and Randall, W. A., THIS JOURNAL, **39**, 185(1950).
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(4) Mason, R., Kice, P., and Caffery, E. L., personal communication.
(5) Musselman, M., personal communication.

The Stabilization of Albumin During the Ultraviolet Irradiation of Plasma*

By JOHN H. HINK, Jr., and FREDERICK F. JOHNSON†

The ultraviolet irradiation of plasma as a means of destroying the serum jaundice virus has not been used when the plasma is to be fractionated into its clinically useful components. This is because the albumin fraction is so denatured by irradiation that its stability is seriously reduced. If 0.04 *M* sodium caprylate is added to the plasma before irradiation, the albumin, as well as the other fractions, can be recovered from the plasma apparently unharmed and sufficiently free of residual caprylate. Data are given to indicate that the presence of 0.04 *M* caprylate does not interfere with the bactericidal and viricidal action of the ultraviolet.

THE POOLING of human plasma from a large number of bleedings prior to fractionation of the plasma could result in a wide dissemination of homologous serum jaundice if effective steps were not taken to destroy any icterogenic virus in each of the end products of plasma fractionation. At the present time, any icterogenic virus in the albumin fraction can be destroyed by heat without undue damage to the albumin (1). There have been no reports of the virus occurring in the γ-globulin fraction. Treatment of fraction I with the nitrogen mustards for the purpose of destroying the virus has been studied, but the safety of injecting proteins which have been reacted with nitrogen mustard is not clearly established. Even though the virus may conveniently be destroyed in these three fractions, the actual process of fractionating plasma may result in the spread of homologous serum jaundice to the fractionation personnel (2). Also, there are numerous other plasma components of potential clinical value, and these other plasma components generally tend to become less stable when separated from the other proteins of plasma. It is becoming increasingly difficult to find means of destroying the icterogenic virus by a special treatment of each new plasma fraction. Clearly, a method of inactivating the virus in plasma without denaturation of the products of plasma fractionation is desirable.

A clinical study of the viricidal effects of irradiation of icterogenic serum has been carried out by Blanchard, *et al.* (3), and indicates that the virus of homologous serum jaundice is effectively inactivated by the proper ultraviolet irradiation of the serum. Although we have noted no gross changes in the antibodies or other proteins of plasma following the ultraviolet irradiation of plasma, we have found that when purified albumin is prepared from irradiated plasma the albumin is markedly denatured, as evidenced by instability to high temperatures. Although albumin is one of the most stable of the plasma proteins, it also seems to be one of the components most easily denatured by ultraviolet due to its high absorption of ultraviolet. Albumin is also a protein which must be prepared in a relatively undenatured state due to the stringent stability requirements necessitated by its mode of usage.

In order to irradiate plasma, and then fractionate the irradiated plasma into the clinically useful fractions free of virus, and still obtain albumin of satisfactory stability to high temperatures, experiments were undertaken to find a stabilizing agent which would protect the albumin during irradiation of plasma but which would not stabilize bacteria or virus against the irradiation treatment. It was also necessary to determine if objectionable amounts of the stabilizer were removed during the fractionation procedure.

Luck and his co-workers (4–8) have used the salts of various aliphatic and aromatic acids to prevent marked denaturation of albumin solutions when the solutions were subjected to heat or urea under conditions which would otherwise cause gross denaturation. Recently (9) they have shown that sodium caprylate will stabilize solutions of albumin against ultraviolet denaturation and also will minimize the increasing absorption of ultraviolet by albumin undergoing irradiation. Strong (10) reported that the presence of tryptophane, the most common of the albumin stabilizers, has been found to enhance the denaturing effect of ultraviolet on albumin. The work of Luck suggested that the albumin of plasma might be stabilized against ultraviolet irradiation by the addition of sodium caprylate to the plasma prior to irradiation.

* Received May 7, 1951, from the Research Division, Cutter Laboratories, Berkeley, Calif.
Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.
† The authors are indebted to Karol Hok for carrying out the measurements on mumps virus.

METHODS

Irradiation

The irradiation equipment used for the irradiation of plasma prior to fractionation consisted of a G36T6 Westinghouse Sterilamp with 86.5% of its effective ultraviolet output impinging on a rotating film of plasma, with a calculated average thickness of 0.2 mm., flowing at 180 ml. per minute in a rotating stainless steel cylinder of 2 in. diameter. The surface exposure time of the flowing plasma was 4.7 seconds. The lamp was rated to deliver 85–95% of its output at 2537 Å., and had an intensity of 7,000 micro-watts per cm.^{2} at 0.20 amp. Calculations using incident light show an energy dosage of 2.5 joules per ml. This apparatus was found to cause more denaturation than later apparatus put into operation in our laboratory and described below.

The irradiation equipment used for the studies involving bacterial inactivation in plasma consisted of a G36T6 Westinghouse Sterilamp with 86.5% of its effective ultraviolet output impinging on a rotating film of plasma with a calculated average thickness of 0.14 mm., flowing at 200 ml. per minute in a rotating stainless steel cylinder of 2.78 in. diameter. The surface exposure time of the flowing plasma was about three seconds. The lamp was rated to deliver 85–95% of its output at 2537 Å., and had an intensity of 6,500 micro-watts per cm.^{2} at 0.42 amp. Calculations using incident light show an energy dosage of 2.9 joules per ml. This apparatus was constructed by the John J. Dill Ultra Violet Co., Kalamazoo, Mich.

The irradiation equipment used for the studies involving virus inactivation in albumin solutions was a Hanovia Biosteritron. This apparatus consists of a lamp rated to deliver 85–95% of its output at 2537 Å., with an intensity of 60 to 90 micro-watts per cm.^{2}, coiled around and impinging on a quartz cell 2 x 10 cm. with a thickness of 0.2 mm. A plasma flow rate of 100 ml. per minute was used for the experiments. Calculations using incident light show an energy dosage of about 1.8 joules per ml.

Analytical

Although a method of sodium caprylate analysis is given by Boyer, *et al.* (11), for the concentrations with which they worked, it was found that errors became noticeable when higher concentrations of the fatty acid salt were used. Also, the published method gave satisfactory results only when the sodium caprylate solution did not contain protein. Two modifications of the analytical method for sodium caprylate were worked out and checked for accuracy.

Determination of Sodium Caprylate in Water Solution.—Two milliliters of sample is acidified with 2 ml. of 0.1 *N* HCl (sufficient to change litmus to deep red), shaken with 20 ml. of petroleum ether, and allowed to settle. A 10-ml. aliquot of the ether is evaporated to dryness at room temperature. The residue is dissolved in 10 ml. of 50% ethanol and titrated to phenolphthalein end point with 0.01 *N* NaOH.

Determination of Sodium Caprylate in Protein Solution.—The protein to be analyzed for sodium caprylate is dissolved to a high dilution to aid in dissociating the protein-caprylate complex. A large amount of petroleum ether is used for the same purpose. One gram of protein material is dissolved in 125 ml. of water. Sufficient HCl is added to make the solution definitely acid to litmus. One hundred milliliters of petroleum ether is added to the acidified solution and the mixture is shaken vigorously. The emulsion which forms is broken by centrifugation at 2,500 r. p. m. at −5°. As the emulsion breaks, the water is frozen. The ether is then decanted and evaporated at room temperature. The residue is dissolved in 10 ml. of 50% ethanol and titrated to phenolphthalein end point with 0.01 *N* NaOH.

To check the accuracy of the method, a 25% solution of serum albumin was prepared to contain 0.02 *M* sodium acetyltryptophane and 0.04 *M* sodium caprylate. The solution was frozen and lyophilized. The dry material was then analyzed for sodium caprylate by the method described above. An amount was found equivalent to 0.038 *M* sodium caprylate in the original solution, and this degree of accuracy was considered adequate for our purpose.

Measurement of Caprylate-Protein Binding

Two methods were used for determining the amount of sodium caprylate bound to plasma proteins.

Ultrafiltration.—Plasma containing a known concentration of sodium caprylate was ultrafiltered through Visking sausage casing (held between two pieces of Whatman No. 50 paper in a small Hormann filter press) by the application of 30 lb. air pressure. The filtrate was analyzed by the method for sodium caprylate in water solution. Plasma solutions of sodium caprylate were ultrafiltered at two different *p*H values. To estimate the passage of unbound sodium caprylate through the ultrafilter, a 0.1 *M* solution of sodium caprylate in water was ultrafiltered. The unfiltered solution analyzed 0.0948 *M* and the ultrafiltrate analyzed 0.0906 *M*. When plasma solutions of sodium caprylate were ultrafiltered, the difference between the molarity of sodium caprylate found in the ultrafiltrate and that known to be added to the plasma was calculated as the amount bound at the concentration added.

Equilibrium Dialysis.—Two types of equilibrium dialysis experiments were set up. In the first type, plasma solutions were prepared of known sodium caprylate molarity, one set at *p*H 7.6 and the other at *p*H 9.4. Ten milliliters of caprylate-plasma solutions were placed in small Visking sausage casings. After being sealed, the sausage tubes were placed in test tubes of a diameter twice that of the inflated sausage casings, and 10 ml. of distilled water was added to each test tube. The tubes were then stoppered and shaken gently at a temperature of 3° for five days. The liquid on the outside of the membrane (about 8 ml.) was then analyzed for caprylate. The difference between the molarity of sodium caprylate found and one-half of that known to be added was calculated as the amount bound at one-half the concentration added. In the second type of equilibrium dialysis experiments, the plasma was placed in the sausage casings as above, but the sodium caprylate was added in known amounts to the 10 ml. of water placed on the outside of the bag.

After five days of shaking at 3°, the liquid on the outside of the membrane (again about 8 ml.) was analyzed as above.

Fractionation of Plasma

Five pools of human plasma were fractionated by method 6 of Cohn (12). Each pool had a volume of about 3,200 ml. A caprylate-fortified 0.04 *M* irradiated plasma pool and an unfortified control pool were fractionated at one time, and the other three, 0.01, 0.02, and 0.03 *M* caprylate-fortified irradiated plasma pools, were fractionated at another time. The purified sodium caprylate was dissolved in the plasma pools by slow swirling for about ten minutes. The pools were irradiated as previously described. The irradiation equipment was cleaned between each pool by passing 0.9% NaCl through the equipment. The first two pools were frozen and allowed to stand at −30° for three days prior to fractionation. The latter three pools were fractionated immediately following irradiation. These three pools were also subfractionated to fraction II (γ-globulin) by the method 9 of Oncley (13). The fractionations were carried out without difficulty. The only differences attributable to the sodium caprylate were the requirement of more acid buffers to attain the required *p*H values for fraction I and fractions II and III, and the characteristic odor of caprylic acid in the centrifugal effluents.

Measurement of Albumin Stability

Each dry albumin fraction from caprylate-fortified and unfortified plasma was dissolved as a 25% solution in distilled water. To each solution was added sodium acetyltryptophane and sodium caprylate to a final concentration of 0.02 *M* of each. Each solution was adjusted to *p*H 7.0 by the addition of sodium carbonate and was sterilized by filtration through a Hormann D-8 pad. The filtrates were filled into special nephelometer vials and the filled vials were heated at 60° for ten hours. Turbidimetric methods were used as a measure of albumin denaturation at 60° and 57°. The special photoelectric tyndallometer was designed by Mueller and Rado [Scatchard (14)] and measures scattered light at an angle of 90°. This tyndallometer also measures transmitted light by which means the decrease in percentage transmission of light due to any development of color in the albumin solution can be measured and applied as a correction to the measurements of scattered light. The turbidity readings are expressed as "nephelometric units." The unit is approximately 1% of the turbidity of a standard of absolute turbidity 0.0193. This is the same unit as employed by Scatchard (15).

RESULTS

Binding of Sodium Caprylate and Plasma Protein.—Boyer, *et al.* (11), found the amount of sodium caprylate bound in an albumin solution to be dependent upon *p*H but not upon temperature within the range studied (3–25°). They found that the binding of caprylate to albumin followed the law of mass action and postulated some theories to explain the binding.

Using the methods previously described, we measured the binding of sodium caprylate with the protein of undiluted citrated plasma at various concentrations of caprylate and obtained the results shown in Fig. 1. Sodium caprylate was not added in amounts sufficient to show a maximum caprylate-plasma protein binding. However, a distinct change in slope at approximately 0.04 *M* added sodium caprylate indicated that at this point the first binding site may be approaching saturation. At 0.04 *M* added sodium caprylate, the bound sodium caprylate is equivalent to approximately 13 moles per mole of albumin. Neither the *p*H nor the temperature seemed to appreciably affect the binding with plasma protein within the ranges investigated.

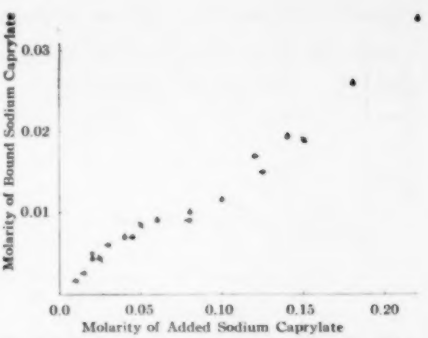

Fig. 1.—The binding of sodium caprylate by human plasma protein at increasing concentrations of sodium caprylate. ○ Ultrafiltration at *p*H 9.4, 25°. ◐ Ultrafiltration at *p*H 7.6, 25°. ◑ Dialysis at *p*H 7.6, 3°. ◔ Dialysis at *p*H 9.4, 3°. ◕ Dialysis at *p*H 9.4, 3°, with sodium caprylate added on outside of bag.

Albumin Prepared from Caprylate-Fortified Irradiated Plasma.—The caprylate-protein binding data of Fig. 1 suggested the trial of 0.04 *M* caprylate as an albumin stabilizer during irradiation. The methods of fortification, irradiation, and fractionation were described earlier.

The dry albumin fractions obtained from the caprylate-fortified and unfortified plasma pools were analyzed for residual caprylate by the method for determinination of sodium caprylate in protein solution. The results are shown in Table I.

TABLE I.—RESIDUAL CAPRYLATE IN PURIFIED ALBUMIN

Cap. Concn. in Plasma	Moles Cap./Gm. Albumin	Cap. Concn. in 25% Alb. Soln.
0.01 *M*	2.86×10^{-4}	0.0082 *M*
0.02 *M*	3.28×10^{-4}	0.0094 *M*
0.03 *M*	3.45×10^{-4}	0.0098 *M*
0.04 *M*	3.50×10^{-4}	0.010 *M*

The caprylate remaining bound to the albumin is shown also as the molarity of caprylate in a 25% albumin solution since the allowable concentrations of albumin stabilizers are commonly expressed in that fashion. At a level of 0.04 *M* caprylate in plasma, about 20% of that bound to plasma proteins, or about 4% of that added to plasma, is recovered with the albumin. If the albumin is

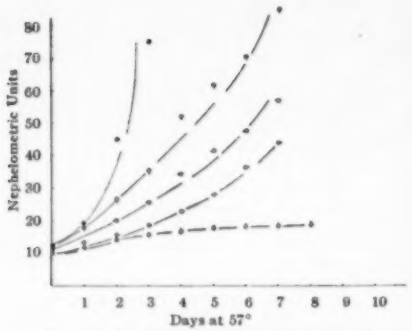

Fig. 2.—57° stability of albumin fractionated from sodium caprylate fortified irradiated plasma and initially heated ten hours at 60°. ◊ from 0.04 *M* caprylate fip, ○ from 0.03 *M* caprylate fip, ◇ from 0.02 *M* caprylate fip, ♀ from 0.01 *M* caprylate fip, ● from non-fip; fip = fortified irradiated plasma.

washed with anhydrous acetone, the residual caprylate can be further reduced to 0.55×10^{-6} moles per Gm. of albumin.

Stability measurements were made on each 25% albumin solution prepared from caprylate-fortified and unfortified irradiated plasma. Following an initial heat treatment of ten hours at 60°, the turbidity of each albumin solution was determined in the nephelometer. The solutions were then placed at 57° and nephelometric readings were made daily. In Fig. 2, the nephelometric units are plotted against days at 57° for albumin prepared from unfortified irradiated plasma and for albumin prepared from irradiated plasma to which 0.01 *M*, 0.02 *M*, 0.03 *M*, and 0.04 *M* sodium caprylate had been added prior to irradiation.

Albumin is considered to be of satisfactory stability if it reads not more than 15 nephelometric units after ten hours at 60° and if the nephelometric units increase by no more than 20 during a subsequent fifty hours at 57°. The albumin prepared from 0.04 *M* caprylate-fortified irradiated plasma and subsequently stabilized with 0.02 *M* sodium acetyltryptophane and 0.02 *M* sodium caprylate is approximately as stable to high temperatures as is albumin prepared from unirradiated plasma and subsequently stabilized in the same manner.

γ-Globulin Prepared from Caprylate-Fortified Irradiated Plasma.—Lyophilized samples of γ-globulin (fraction II) prepared from the 0.01 *M*, 0.02 *M*, and 0.03 *M* caprylate-fortified irradiated plasma were analyzed for residual caprylate, and no detectable amount of caprylate was found in any sample. The globulin from each fractionation was then dissolved in 0.3 *M* glycine to form a 16% protein solution and sterilized by filtration through a D-6 Hormann pad. A portion of each filtrate was analyzed for nondialyzable nitrogen by the Kjeldahl method, and the actual protein concentrations after filtration were calculated. Another portion of each filtrate was tested for thermal stability at 57°. The resulting data is shown in Table II. A γ-globulin solution of satisfactory stability will show a gelation time of not less than four hours at 57°. A gelation time of thirteen to fifteen hours

TABLE II.—GELATION TIME OF γ-GLOBULIN PREPARED FROM CAPRYLATE-FORTIFIED IRRADIATED PLASMA

Cap. Concn. in Plasma	Globulin in Soln., %	Gelation Time at 57°, Hr.
0.01 *M*	13.5	13.5
0.02 *M*	14.0	14.2
0.03 *M*	16.0	15.0

is about average for a good quality γ-globulin prepared from unirradiated plasma.

Fraction I Prepared from Caprylate-Fortified Irradiated Plasma.—Each fraction I (fibrinogen-containing fraction) was lyophilized, dissolved in pH 6 citrate buffer, filtered, and the filtrate tested for clottable protein. When the filtrate is clotted by the addition of thrombin and Ca^{++}, the nitrogen in the washed clot expressed as the per cent of total nitrogen is assumed to represent the per cent clottable protein. All fraction I samples showed approximately a 45% clottable protein, which is the value usually obtained for fraction I prepared from nonirradiated plasma by the method of Cohn and which has been lyophilized and filtered. Fraction I prepared from caprylate-fortified irradiated plasma appears suitable for the preparation of fibrin foam and fibrinogen injections.

Effect of Sodium Caprylate on the Destruction of A. Aerogenes and Mumps Virus by Ultraviolet Irradiation.—Citrated plasma was seeded with *Aerobacter aerogenes* (1 million to 10 million organisms per ml.), and then sodium caprylate was added to a concentration of 0.04 *M*. A portion of the caprylate-fortified contaminated plasma was then irradiated as previously described. Samples of the plasma were plated out under standard conditions. Plates from the nonirradiated plasma, both before and after the addition of sodium caprylate, showed many typical colonies, whereas the irradiated plasma was sterile.

As there is no known method at this time of cultivating the virus of homologous serum jaundice outside of the human host, it was not practical to conduct a similar experiment to determine if sodium caprylate stabilizes the virus of homologous serum jaundice against ultraviolet irradiation. Mumps virus was selected for study in its place. However, the plasma pools were found to destroy mumps virus so rapidly that no control comparisons could be made. Accordingly, a 7% solution of albumin was used in place of plasma. The use of an albumin solution probably constitutes a more rigorous test than the use of plasma, since the high absorption of ultraviolet by albumin would, if anything, decrease the viricidal action of the ultraviolet. Sodium caprylate was added to two of the 7% albumin solutions to give a concentration of 0.04 *M*. Both solutions were seeded with mumps virus (ATC strain) to give a 1:100 dilution of the chick embryo allantoic fluid supernatant. One of the virus-containing solutions was irradiated as previously described, and the other solution was used as a control. Other controls are indicated in Table III.

Nine-day-old embryonated eggs were inoculated allantoically with 0.1-ml. amounts of appropriate dilutions of the irradiated and unirradiated solutions and were then incubated at 35° for five days. The allantoic fluid was harvested separately from each

TABLE III.—EFFECT OF SODIUM CAPRYLATE ON ULTRAVIOLET INACTIVATIO OF MUMPS VIRUS IN ALBUMIN SOLUTION

Description of Sample	Dilutions (in Broth)	No. of Eggs	CCA Agglutination in Eggs No. 1	2	3	4	5	E. I. D$_{50}$ per 0.1 Ml.
Albumin + Virus + UV	10^{0}	5	−	−	−	−	−	0
	10^{-1}	5	−	−	−	−	−	
	10^{-2}	5	−	−	−	−	−	
	10^{-3}	5	−	−	−	−	−	
	10^{-4}	5	−	−	−	−	−	
Albumin + Virus + Caprylate + UV	10^{0}	5	−	−	−	−	−	0
	10^{-1}	5	−	−	−	−	−	
	10^{-2}	5	−	−	−	−	−	
	10^{-3}	5	−	−	−	−	−	
	10^{-4}	5	−	−	−	−	−	
Albumin + Virus	10^{0}	2	+	+				$10^{3.4}$
	10^{-3}	5	+	+	+	+	−	
	10^{-4}	5	−	−	−	−	−	
	10^{-5}	5	−	−	−	−	−	
	10^{-6}	5	−	−	−	−	−	
Albumin + Virus + Caprylate	10^{0}	2	+	+				$10^{4.5}$
	10^{-3}	5	+	+	+	+	+	
	10^{-4}	5	+	+	+	+	+	
	10^{-5}	5	−	−	−	−	−	
	10^{-6}	5	−	−	−	−	−	
Broth + Virus	10^{0}	3	+	+	+			$10^{3.5}$
	10^{-1}	3	+	+	+			
	10^{-2}	3	+	+	−			
	10^{-3}	5	+	+	+	−	−	
	10^{-4}	5	+	+	−	−	−	
	10^{-5}	5[a]	+	+	−			
	10^{-6}	5	−	−	−	−	−	
	10^{-7}	5	−	−	−	−	−	
	10^{-8}	5	−	−	−	−	−	

[a] Two embryos were found dead and were discarded.

egg, diluted 1:2, and tested for the presence or absence of mumps virus by the chicken cell agglutination method. The 50% end points were calculated according to the method of Reed and Muench (16). The results are given in Table III.

DISCUSSION

Duggan, Giese, and Luck (9) postulated several mechanisms by which caprylate and other similar anions are able to "stabilize" bovine albumin against various changes caused by the denaturing action of ultraviolet irradiation. We offer no further evidence to modify these theories, but do show that sodium caprylate will stabilize the albumin of plasma against the denaturing action of ultraviolet as measured by the turbidity of albumin subjected to 60° and 57° heat. The stabilizing effect of 0.04 *M* sodium caprylate in the irradiation of plasma protects the albumin to such a degree that it compares favorably in heat stability to albumin prepared from nonirradiated plasma.

The stabilizing action of sodium caprylate on albumin is known to be somewhat specific, and Boyer, Ballou, and Luck (11) mentioned that sodium caprylate accelerated the heat denaturation of γ-globulin. Although it is apparently true that caprylate will accelerate the heat denaturation and gelation of a 16% γ-globulin solution, we have found that caprylate dissolved in plasma subjected to ultraviolet irradiation does not measurably harm the γ-globulin of plasma. The removal of substantially all sodium caprylate is accomplished during the fractionation of the plasma, and a solution of γ-globulin prepared from caprylate-fortified irradiated plasma is as stable against heat denaturation as is γ-globulin prepared from a noncaprylate-treated nonirradiated plasma.

Previous but unpublished data show that the irradiation of plasma without caprylate does not measurably affect the pertussis agglutinin or diphtheria antitoxin. New data, not included in this report for the sake of brevity, indicates that irradiation and caprylate fortification does not destroy the mumps HI antibody.

The effect of the sodium caprylate stabilizer on bacteria and virus was determined to assure that caprylate did not stabilize these infective agents against the denaturing effect of ultraviolet irradiation at 2537 Å. The results show that the irradiation inactivates both the test bacteria and the test virus when the caprylate stabilizer is present in a concentration of 0.04 *M*.

Using smaller amounts of sodium caprylate greatly reduces the stabilizing effect on albumin. The greater the concentration of added caprylate, the greater is the concentration in the purified albumin fraction. Yet it is found that when an effective amount of sodium caprylate is added to the plasma and the plasma is fractionated, a resulting 25% albumin solution contains only 0.01 *M* caprylate, and this amount can be further reduced. However, as 0.02 *M* caprylate is commonly used at present as a stabilizer for 25% albumin solutions, this further reduction in caprylate content is not required.

The stabilization of plasma albumin during ultraviolet irradiation makes possible the fractionation of irradiated plasma into its components of present

clinical usefulness: albumin, γ-globulin, fibrinogen, and thrombin. It is quite possible that other plasma components of potential clinical value are harmed or destroyed by the action of either ultraviolet or caprylate. Tests conducted thus far indicate that neither the irradiation nor caprylate destroy the plasma cholinesterase or iron-binding globulin (transferrin).

SUMMARY

1. Albumin prepared from ultraviolet irradiated human plasma is denatured to an extent easily measured by its decreased stability at 57°.

2. The addition of sodium caprylate to plasma prior to irradiation substantially decreases or eliminates the denaturation of albumin by ultraviolet irradiation.

3. Under the conditions of these experiments, the presence of 0.04 *M* sodium caprylate in plasma does not interfere with the destruction of *Aerobacter aerogenes* and mumps virus by ultraviolet irradiation.

4. The presence of 0.04 *M* sodium caprylate during the irradiation of plasma causes no measurable deleterious effect on γ-globulin or fibrinogen.

5. When plasma containing 0.04 *M* sodium caprylate is irradiated and fractionated, approximately 4 per cent of the caprylate is recovered bound to the albumin, and no detectable amounts of caprylate are bound to the other clinically useful fractions.

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The Metabolism of Sorbitol*,†

By ARNE N. WICK, MARY C. ALMEN, and LIONEL JOSEPH

Studies on the metabolism of sorbitol (labeled with carbon 14) indicate that it is rapidly oxidized when administered by the intraperitoneal route. When sorbitol is given orally a slower rate of oxidation is obtained because of a delayed intestinal absorption. Although sorbitol is converted to glucose in its metabolism, the slow rate of intestinal absorption tends to prevent a high degree of hyperglycemia.

SORBITOL, a hexahydroxy alcohol occurring naturally in many fruits, is produced synthetically for commercial purposes by the catalytic reduction of glucose. This alcohol, which has a cool, sweet taste, is becoming more useful in the preparation of candy and other food materials because of its unusual ability to control moisture content. Its derivatives are being used in increasing quantities as solvents, emulsifying and dispersing agents for foods and medicines.

Sorbitol has also been recommended over a period of years as a substitute for sugar in the diabetic diet. In an excellent review on the metabolism of the sugar alcohols, Carr and Krantz (1) express the following opinion regarding sorbitol: "It owes its value in diabetes to the fact that it is capable of being stored as glycogen and that its subsequent depolymerization and utilization fail to supply to the blood a plethora of glucose which would produce hyperglycemia."

Since no information is available regarding the fate of ingested sorbitol except that it is a glycogen precursor, we have prepared radioactive sorbitol for metabolism studies. The degree of oxidation (to CO_2) of sorbitol and the extent to which sorbitol carbon atoms are incorporated

* Received July 9, 1951, from the Scripps Metabolic Clinic, La Jolla, Calif.
† This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

into liver and body glycogen have been determined. The rate of intestinal absorption of sorbitol after oral feeding was also examined. For comparison, similar studies were carried out using C^{14}-labeled glucose.

METHODS

Preparation of Radioactive Sorbitol.—The radioactive sorbitol was prepared by the catalytic reduction of C^{14}-labeled glucose with Raney nickel (2). The tagged glucose was uniformly labeled and obtained by the hydrolysis of starch prepared by photosynthesis (3). The sugar alcohol was nonreducing and it was identified as sorbitol by its m. p. and mixed m. p. with an authentic sorbitol sample. The preparation contained no radioactive contaminants when examined by paper chromatography and exposure to X-ray film as described for the sugars (3).

Measurement of C^{14} Radioactivity.—All radioactive determinations were carried out on $BaCO_3$ obtained directly or by dry combustion. In all cases the samples were of infinite thickness (4).

Biological Experiments: Rate of Oxidation.—Male albino rats (100 Gm.) raised on Purina Laboratory Chow were used. The animals were placed in individual metabolic chambers similar to those described by Armstrong, *et al.* (5), immediately after an oral or intraperitoneal injection of the radioactive substance. Sufficient stock food and water were supplied for the experimental period. The expired CO_2 was collected and precipitated as $BaCO_3$ at three-hour intervals for the first twelve hours and at six-hour intervals for the duration of the experiment. The urine was collected as one sample in each run.

The procedure used in the glycogen studies was identical to that employed by Johnston and Deuel (6). Male albino rats (140–160 Gm.) previously fasted forty-eight hours were divided into three groups and each group given C^{14}-labeled sorbitol, C^{14}-labeled glucose, and a combination of plain glucose and $NaHC^{14}O_3$, respectively. These materials were administered intraperitoneally in two doses at three-hour intervals. The total amount injected per 100 $cm.^2$ of body surface was 1.0 ml. of a 25% solution of the test substance. Six hours after the initial injection the animals were sacrificed for liver glycogen and radioactivity determinations. The specific activity (cts./min./mg. carbon) of the radioactive sorbitol and glucose samples used in the glycogen experiments was 1850 and 1880, respectively. Inert glucose and $NaHC^{14}O_3$ were given in order to estimate the degree of CO_2-fixation. The total counts given as $NaHC^{14}O_3$ were the same as in the sorbitol and glucose experiments and represent approximately 15 mg. of $NaHCO_3$ per rat.

Intestinal Absorption.—Female albino rats weighing 210–252 Gm. were used. They were fasted twenty-four hours after removal from a stock diet of Purina Dog chow. Two groups of rats were used with nine rats per group. To each group of nine rats was administered by stomach tube, 300 mg. of radioactive sorbitol and 300 mg. of labeled glucose, respectively. Three animals in each group were sacrificed at one, two, and three-hour periods after the ingestion of the test substance. The intestinal tract and stomach were removed and washed with 50–75 ml. saline. The contents were dried by lyophilization. The intestinal absorption of the labeled compounds was calculated by determining the amount of radioactivity recovered in the intestinal contents.

Samples of blood were also taken at the time of sacrifice for radioactivity measurements.

RESULTS

The rate of appearance of $C^{14}O_2$ in the expired air of the rats given labeled sorbitol and glucose is shown in Fig. 1. The results are the average values for a series of experiments in which the amount of test substance administered to the rats was as follows: glucose I. P., 21 mg.; glucose S. T., 21 and 20 mg.; sorbitol I. P., 5, 17, and 30 mg.; sorbitol S. T., 5, 17, and 186 mg. In all cases the results obtained by any one of the types of experiment were comparable.

The total expired $C^{14}O_2$ recovered in the twenty-four-hour period was 63–79% for sorbitol and 85–90% for glucose. Although sorbitol shows rapid combustion to CO_2 under certain conditions, the quantity oxidized at the end of three hours is not as high as that of glucose when administered by stomach tube. After the oral administration of sorbitol the individual recoveries at this time were 14, 11, and 11%, while the values for glucose were 45 and 67%. When the sorbitol was injected intraperitoneally, the recovery at three hours was 45, 43, and 40%, which is only slightly below that observed for glucose. It thus appears that the decreased oxidation of sorbitol observed at the three-hour period after oral administration is due to its delayed absorption. The C^{14} recovered in the urine for the runs given in Fig. 1 never exceeded 5% of the fed counts.

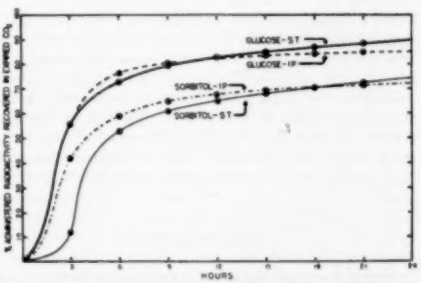

Fig. 1.—Rate of appearance of C^{14} in expired CO_2 after administration of labeled sorbitol and glucose.

The values showing the per cent intestinal absorption of sorbitol and glucose at one-, two-, and three-hour periods after oral ingestion are presented in Table I. These data confirm the conclusion reached from the expired CO_2 data that sorbitol is more slowly absorbed than glucose. Although there was considerable variation, substantial amounts of sorbitol remained in the intestinal tract three hours after feeding. Glucose is almost quantitatively absorbed in one hour under identical experimental conditions.

TABLE II.—THE EFFECT OF ADMINISTRATION OF SORBITOL AND GLUCOSE ON LIVER AND BODY GLYCOGEN AND INCORPORATION OF CARBON 14

Substance Injected	Liver			Carcass		
	Glycogen, %	Glycogen from Compd., %	Glycogen S. A.	Glycogen, %	Glycogen from Compd., %	Glycogen S. A.
C^{14} Sorbitol	4.0 ± 0.4	42 ± 2	772 ± 33	0.22 ± 0.04	9 ± 0.8	162 ± 16
C^{14} Glucose	4.5 ± 0.3	37 ± 1	699 ± 23	0.26 ± 0.03	18 ± 0.9	339 ± 18
Glucose + $NaHC^{14}O_3$	2.9 ± 0.2	...	164 ± 9	0.26 ± 0.02	...	43 ± 2.6

The rapid rate of glucose absorption in contrast to that observed for sorbitol is reflected as expected in the high radioactivity content of the whole blood (Table I) in the glucose-treated animals. On the other hand, the slow absorption of sorbitol prevents sorbitolemia. A difference in the rate of absorption between sorbitol and glucose can be expected since it was shown long ago by Cori (7) that the intestinal membrane is possessed of a high degree of selective permeability for sugars.

TABLE I.—DATA SHOWING RATE OF INTESTINAL ABSORPTION AND RADIOACTIVITY PER CC. OF BLOOD AFTER FEEDING LABELED SORBITOL AND GLUCOSE

Substance Fed	No. of Rats	Sacrifice Time After Inj. (Hr.)	% C^{14} Absorbed (Av.)	Cts./Cc. Whole Blood (Av.)
Sorbitol	3	1	75	34
	2	2	66	26
	3	3	84	14
Glucose	3	1	97.1	416
	3	2	99.1	372
	3	3	98.9	125

That sorbitol is a precursor of glycogen is clearly demonstrated by the radioactivity data given in Table II which show that sorbitol is comparable to glucose in its ability to form liver glycogen. By calculating on the basis of carbon from the S. A. figures, it is found that 42 and 37% of the liver glycogen came from the fed sorbitol and glucose, respectively. Since the S. A. of the samples from the inert glucose and $NaHC^{14}O_3$ experiments are approximately one-fifth that of the test runs, it may be assumed that 20% of the total C^{14} content of the glycogen samples was incorporated by CO_2 fixation. It is realized, of course, that the control runs for CO_2 fixation can at best only approximate normal conditions.

DISCUSSION

There is considerable information indicating that sorbitol can be a precursor of liver glycogen (6, 8–10). Our positive evidence with radioactive sorbitol confirms these findings and also shows that sorbitol enters into carcass glycogen. It seems most likely that the metabolism of sorbitol resembles that of glucose after its conversion to glucose, which we assume to take place in the liver. We have previously administered sorbitol to a liverless animal (11) and found no $C^{14}O_2$ in the expired air. It is therefore believed that the C^{14} of the carcass glycogen resulted from the floating glucose or glucose intermediates liberated by the liver cells.

One would assume that the feeding of a glycogenic substance such as sorbitol would elevate the blood sugar. However the literature is controversial in this matter, as shown in the review by Carr and Krantz (1). Our results show that the delayed oxidation of sorbitol in comparison with glucose after oral feeding is due to a decreased rate of intestinal absorption and, to a less extent, to the extra step needed for the conversion of sorbitol to glucose by the liver. These results support the view that the formation of glucose from sorbitol in a normal animal is gradual and evenly distributed and thus incapable of producing a significant hyperglycemia.

SUMMARY

The total oxidation of C^{14}-labeled sorbitol over a twenty-four-hour period approaches that of labeled glucose when given to fed normal rats. Sorbitol administered orally is oxidized at a much slower rate for the first three hours. The slower rate of oxidation was shown to be due chiefly to delayed intestinal absorption.

C^{14}-Labeled sorbitol and glucose produce equal amounts of liver glycogen when given to fasted rats; the amount of incorporation of C^{14} in the glycogen is the same with the two compounds. It is believed that the metabolism of sorbitol proceeds through glucose.

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Salivary Amylase: Its Activity and Stability with the Evidence for the Existence of a Labile Fraction*,†

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The activity of salivary amylase is due to at least two different amylases. One enzyme that has amylase activity is stable to dissolved oxygen, while another enzyme that has amylase activity is labile to dissolved oxygen. The irregularities in the activity of salivary amylase in the same individual are not as great as the literature indicates. Uniform salivary amylase activity in the same individual may be shown by one of two different methods of analysis. In one method the sample of saliva is oxygenated before the analysis is made. This gives the salivary amylase activity of the oxygen-stable form. In the other method the sample of saliva is protected against oxygen. This gives the amylase activity of both the oxygen-stable and oxygen-labile forms. Irregular results in the assays of salivary amylase are obtained with the ordinary methods of analysis in which the inactivating effect of oxygen is not considered.

SALIVARY amylase has generally been regarded as being composed of one amylolytic enzyme. Kühn (1) in 1925 classified this enzyme as being of the α-type. Meyer, Fischer, Staub, and Bernfeld (2) isolated and then crystallized a stable pure form of an α-amylase from human saliva. Later work of Bernfeld, Staub, and Fischer (3) showed that solutions of this crystalline material are stable. However, in an earlier paper (4), it was noted that a decrease in activity took place in fresh saliva after a dilution had stood for one hour or more. It was also noted that the saliva deteriorated in each case to about one-half of its original activity, and that after it had reached this level, the activity remained uniform for at least several weeks. It was thought at that time that a labile activator that lost its effectiveness within one hour might have been present in the freshly secreted saliva. The present work was undertaken as an attempt to determine the nature of this labile portion, and the means of preventing its escape or deterioration. It was also noted (4) that storage of saliva at 2° seemed to have the effect of activating salivary amylase. These observations, and the problems of testing the activity of salivary amylase in the presence of various materials, constitute the basis of the investigation reported in this paper.

HISTORY

Observation of the influence of the glutinous component of wheat meal upon the conversion of starch into soluble carbohydrates led Kirchhoff in 1814 to make the first record of the effect of a biochemical catalyst. In 1831 Leuchs (5) first observed the digestive action of human saliva upon starch. The active principle of saliva was named "ptyalin" by Berzelius. Phenomena of this sort were classified as enzymatic activity by Kühn in 1878. As early as 1863 attempts had been made to concentrate and extract the active principle of saliva by adsorption with normal calcium phosphate (6), and later, with aluminum oxide (7, 8). Ninomiya (9) in 1940 obtained an impure but rich product by fractional precipitation with ammonium sulfate. In 1948 Meyer, Fischer, Staub, and Bernfeld (2) isolated and crystallized an α-amylase from human saliva by acetone-ammonium sulfate fractionation at low temperatures.

The effect of sodium chloride on the activity of salivary amylase was noted in the works of Nasse (10), Chittenden and Ely (11), Kübel (12), and Wohlgemuth (13). In 1911 Bang (14) observed that dialysis decreased the activity of salivary amylase, and that the activity could be restored by sodium chloride. Kübel (12) and Wohlgemuth (13) had observed previously that the bromide, iodide, nitrate, and chlorate of sodium also caused varying degrees of activation.

The influence of *p*H and of temperature upon the activity of salivary amylase was investigated in 1922 by Ernström (7). In 1925 Kühn (1) found that salivary and pancreatic enzymes act upon starch so as to give a high yield of α-maltose. He further observed that enzymatic extracts from either sprouted or unsprouted grains so act upon starch as to produce primarily β-maltose. To indicate the difference in the nature and behavior of these two classes of enzymes, he named the former class the α-type of amylase and the latter class the β-type of amylase.

Recently, (1946), Anker and Vonk (15) assert they have also found the β-type in saliva. The hydrolysis of a small part of the starch to glucose, beyond the maltose stage of digestion, has been attributed by Haworth, Kitchen, and Peat (16) to the presence of a maltase in the saliva. At this stage of investigation it appears that saliva contains at least three different carbohydrases: namely, α-amylase, β-amylase, and maltase. In this paper the stability and activity of an oxygen-labile amylase is reported.

*Received April 30, 1949, from the Massachusetts College of Pharmacy, Boston, Mass.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Edward V. Svedres in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
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EXPERIMENTAL

Method of Assay.—Saliva was collected in a small graduated cylinder until at least 2 cc. of liquid saliva was obtained. Two cubic centimeters of the liquid portion was removed with a graduated pipette, transferred into a volumetric flask, and diluted with sufficient distilled water to yield a 2% solution of unfiltered saliva. The contents of the flask were throughly mixed by inversion. The assay was then conducted by the method described by Bauer and Martin (4) with this modification: 2 cc. of the 2% solution of unfiltered saliva was used without being centrifuged. This modification was made after investigation had shown that centrifuging of a saliva dilution in no way affected the activity or stability of the amylolytic content.

The Effect of Air Under Various Conditions of Temperature and Storage Upon the Amylolytic Activity of Saliva.—*A. Undiluted Saliva.*—It was found that saliva which had been collected in a small test tube, had been stoppered, and had been permitted to stand with its natural supernatant layer of froth, retained its maximum amylolytic activity as long as the supernatant layer of froth was not dissipated. Direct aeration of the saliva, however, resulted in the loss of about half of its amylolytic activity which is shown in the experimental work reported in this paper. It was further found that unaerated and undiluted saliva at 0° maintained its maximum activity for a period of at least one week, whereas at room temperature the froth gradually dissipated and apparent aeration took place with ensuing loss of about half of the activity. It was noted, however, that undiluted saliva in vessels open to air retained its froth for some hours, and at the same time, its maximum activity. The previously reported preservative action of toluene on enzyme solutions (17) may be in part due to the exclusion of air and not exclusively to the reported antiseptic action of toluene against microorganisms. It is reasonable to believe, therefore, that the froth supernatant to liquid saliva preserves the amylolytic activity by the exclusion of air, and that the protective action of toluene, previously used in preservation (4), served in a similar manner.

In the previously reported work (4) it was noted that samples of saliva, stored at 2° for one week without a preservative, seemed as a result to have become "reactivated." In the present work it is shown that there is no reactivation, for the saliva had merely retained its maximum activity through the protective action of the froth. It is also of note that warming of undiluted saliva at 60° for one hour destroys all amylolytic activity, even when there is an ample supernatant layer of froth.

B. Diluted Saliva.—In the work with the saliva assays, it was noted that when dilutions were assayed after having stood for about one hour at room temperature, the time of digestion in the assays doubled, indicating a decrease in activity of the salivary amylase. At this time the assays were all being conducted with ordinary distilled water for dilution. Assays were made of dilutions preserved both at 0° and at room temperature in order to determine the effect of low temperatures upon the preservation of the activity of saliva in these dilutions. It was noted in each assay that the salivary amylase activity of these dilutions deteriorated to half of its initial activity. The effect of storage upon the salivary amylase activity of the dilutions of saliva with ordinary distilled water and without preservatives is shown in Table I.

TABLE I.—THE DIGESTION TIME IN MINUTES OF SALIVA DILUTED WITH ORDINARY DISTILLED WATER AND STORED WITHOUT PRESERVATIVES

	Sample of Saliva		
	1 (2 Samples)	2 (5 Samples)	3 (3 Samples)
Immediate assay	8	10	8
R. T. after 1 hr.	17	19	17
0° after 1 hr.	17	..	17
R. T. after 4 days	..	..	18
0° after 4 days	..	20	18
R. T. after 9 days	..	..	18
0° after 9 days	..	20	17

The next step was to determine the length of time it took saliva to lose one-half of its salivary amylase activity. It had been noted in the course of these assays that after the saliva dilutions had lost one-half their amylase activity, the remaining amylase even without preservatives was extremely stable for several weeks at both room temperature and 0°. It was found that the loss in activity starts immediately after dilution and is complete in fifteen minutes. These assays are presented in Table II.

TABLE II.—DETERMINATION OF THE LENGTH OF TIME IT TAKES SALIVA TO LOSE ABOUT ONE-HALF OF ITS SALIVARY AMYLASE ACTIVITY AFTER DILUTIONS WITH ORDINARY DISTILLED WATER

Time After Dilution, Min.	Digestion Times of Assays, Min.			
	Sample A	Sample B	Sample C	Sample D
2	8½	10	10	8½
5	..	11	11	14
10	..	12	12	15
15	14	13	13	16
20	..	13	..	..
25	..	13	..	..
30	14	13	..	..
45	14	13	..	..
60	14	13	13	16

The Evidence for the Existence of a Labile Fraction of Salivary Amylase.—Deterioration of the initial activity of salivary amylase has been shown in the preceding sections of this paper. As has been noted, this inactivation of amylase occurs upon the aeration of saliva, or its dilution with ordinary distilled water. The deterioration seemed, from these observations, to have been caused by a gaseous component of the air, which might also have been dissolved in distilled water. A series of assays were undertaken in which freshly boiled and cooled distilled water was used for dilutions. It was then found that the initial activity of the saliva dilution was retained and that no inactivation occurred when the dilution was kept for one hour at room temperature. Extreme caution had to be employed to prevent aeration of the dilution and digestion mixture. Along with these assays, another series was run, in

which freshly boiled and cooled distilled water, through which pure oxygen had been bubbled, was used. Upon immediate assay these dilutions gave about one-half the activity of those kept free of oxygen. The oxygen-free dilutions, which had maintained undiminished activity, were then subjected to oxygenation. Upon immediate assay after oxygenation they lost about one-half of their previous activity. Continuous bubbling of oxygen for one hour longer resulted in no further change in activity. On the basis of these observations, it appears that there are at least two different amylases in the composition of the salivary amylase of human saliva. One of these appears to be an oxygen-labile type, while the other is an oxygen-stable type.

It is therefore reasonable to interpret previous inactivations of saliva dilutions as having been caused by oxygen, normally dissolved in distilled water which has stood for a period of time in contact with the atmosphere, or under a pocket of air in a container. A re-examination of previous work in the light of these observations shows that the irregularites observed in the activity of the same individual's saliva may not be as great as the literature (4, 18) indicates.

In Table III, assays of oxygen-free dilutions and of some oxygenated dilutions are shown. It is of interest that an immediate assay of saliva diluted with ordinary distilled water gives an amylase activity which lies between that of the immediate assay in which oxygen-free distilled water is used and that of the assay in which oxygenated distilled water is used. The inactivating effect of oxygen appears to be quantitative, and in the intermediate values to be proportional to the amount of the dissolved oxygen. This offers a plausible reason why it usually requires about fifteen minutes for the saliva to lose one-half of its activity when diluted with ordinary distilled water. It further explains why the use of freshly oxygenated, recently boiled and cooled, distilled water immediately inactivates the oxygen-labile fraction. It also explains why the use of ordinary distilled water gives irregular results, for the amount of oxygen dissolved in the distilled water is not always the same.

The effect of dissolved oxygen on the activity of salivary amylase, as shown in Table III, has been confirmed repeatedly in this investigation. The slight variations that occurred in some assays can be explained by the fact that it is difficult to make a saliva dilution without introducing some air that may cause an inactivation of a portion of the salivary amylase.

Comparison of the Activity of Salivary Amylase, Before and After Prolonged Continuous Mastication.—Various materials, such as chewing gum and yellow wax, were masticated over periods of time up to four hours, in order to determine the effect of possible jaw fatigue on the activity of salivary amylase in the saliva secreted. In all cases the saliva secreted during five minutes of active mastication was kept in the mouth until collected. Two cubic centimeters of the liquid saliva was taken, diluted, and assayed. Though ordinary distilled water was employed for dilution, the immediate assays were representative of maximum activity, for assays of the dilutions after an hour's standing at room temperature showed a loss of half of the initial activity.

TABLE III.—THE EFFECT OF OXYGEN UPON THE ACTIVITY AND STABILITY OF SALIVARY AMYLASE

Dilution of Fresh Saliva Sample	Digestion Times of Assays, Min.	
	Immediate Assay	Assay 1 Hr. After Dilution
With recently boiled distilled water	5	5
With ordinary distilled water	7	10
With oxygenated recently boiled distilled water	10	10
With oxygenated ordinary distilled water	10	10
With recently boiled distilled water; oxygenated after dilution	10[a]	10[b]

[a] Oxygenated by slow bubbling of pure oxygen for two minutes.
[b] Oxygenated by slow bubbling of pure oxygen for one hour.

The immediate assays in each case in which the saliva was collected after varying periods of mastication can then be construed as representing initial and maximum amylolytic activity of the saliva. The results of these assays are presented in Table IV. It was found that vigorous mastication up to four hours, by which the jaws were greatly fatigued, had no effect upon the activity of salivary amylase. In Table IV, sample A is the saliva collected after the mastication of chewing gum; sample B is the saliva collected after the mastication of yellow wax. The physical fatigue of the jaws at the end of four hours was so great that the subject was unable to prolong the chewing process.

TABLE IV.—THE EFFECT OF ACTIVE MASTICATION ON THE ACTIVITY OF SALIVARY AMYLASE

Time of Mastication, Hr.	Digestion Times of Immediate Assays, Min.	
	Sample A	Sample B
0[a]	7½	8½
1	7½	9
2	7½	8½
3	7½	8½
4	7	8

[a] Immediate assays of saliva before any mastication.

Determination of the Effect of Penicillin Chewing Troches and Sulfathiazole Gum Upon the Activity of Salivary Amylase.—After it had been determined that mastication alone had no effect upon the activity of salivary amylase, it was thought feasible to determine the effect of some chewing medications upon its activity. Determinations were made of the effect of penicillin chewing troches and of sulfathiazole gum on the activity of salivary amylase. All assays were conducted with freshly boiled distilled water cooled to room temperature. Fresh saliva was collected after two pieces of sulfathiazole gum had been chewed for ten minutes, each piece containing 0.25 Gm. of sulfathiazole. The saliva was diluted and assayed. A similar assay was run with fresh saliva collected after a 20,000-unit penicillin troche had been chewed for fifteen minutes. Controls were run with fresh saliva, not subjected to any medication for comparison. It was found that neither the penicillin nor the sulfathiazole

showed any inactivation of the amylolytic activity of saliva. Assay of the dilutions showed a very slight inactivation of the amylase after it had stood for one hour at room temperature. This slight variation may be ascribed to aeration during the making of the dilution. The results of the assays are tabulated and presented in Table V.

TABLE V.—THE INFLUENCE OF SULFATHIAZOLE GUM AND PENICILLIN CHEWING TROCHES UPON SALIVARY AMYLASE ACTIVITY

Sample	Digestion Times of —Saliva Dilutions, Min.— Immediate Assay	Assay 1 Hr. After Dilution
Fresh saliva control	6½	7
Aerated fresh saliva control	14	14
Fresh saliva from 10 min. chewing of sulfathiazole gum	7	8
Fresh saliva from 15 min. chewing of penicillin troche	7	7

Determination of the Possible Presence of a Labile Fraction in Commercial Preparations Containing Amylolytic Enzymes.—As has already been shown in this paper, saliva contains an oxygen-labile fraction that possesses amylolytic activity. It is therefore desirable to determine the possible presence of any analogous labile fractions in commercial preparations having amylolytic enzyme activity. Pancreatin U. S. P. and malt extract N. F. were tested for amylolytic activity by the official methods of assay (19, 20) with the following modifications: the assays were first run with freshly boiled distilled water, and were then run with oxygenated freshly boiled distilled water for dilutions. At definite intervals of time 0.1 cc. of each digestion mixture was removed and was added to the iodine dilutions described in the official monographs, the lack of a blue or violet color constituting the end point. It was found that the assay times with and without oxygenated water dilutions were the same for each of the substances tested. Tests were also made by the methods of assay for salivary amylase employed in this paper, with the modification that dilute solutions of malt extract and suspensions of pancreatin were substituted for saliva dilutions. It again was found that similar concentrations of the tested substances gave the same times in the assay, conducted in oxygen-free water and in oxygenated water. Further subjection of dilutions of these materials to the bubbling of oxygen through them gave no inactivation of the amylolytic activities.

A freshly prepared pancreatin having three times the amylolytic activity of pancreatin U. S. P. also gave no evidence of any labile amylolytic activity when it was assayed by the methods above.

In the assay for starch digestive power in the U. S. P. monograph of pancreatin (19) it is stated: "Boil a sufficient amount of water for 10 minutes, and cool to room temperature.[1] Use this water for all dilutions hereinafter specifying water."

[1] In the U. S. P., water used for tests is distilled water.

On the basis of the preceding determinations, doubt is cast on the necessity of the U. S. P.'s specifying freshly boiled water for dilution in the assay for amylolytic activity. It has been shown that there is no labile fraction in commercial pancreatin since the amylolytic activity is not diminished by oxygen. Further tests were conducted upon commercially available products, labeled α-amylase and β-amylase. They were tested and were found not to contain any labile fraction that had amylolytic activity subject to oxidation. They were tested by the methods previously described in the testing of malt extract and pancreatin.

The Effect of Some Mild Oxidizing and Reducing Agents on the Amylolytic Activity of Saliva.—*A. Oxygen.*—As has been previously noted, oxygen normally present in distilled water destroys the activity of the labile fraction in fifteen minutes. Freshly oxygenated water, prepared by slowly bubbling oxygen U. S. P. for two to three minutes through freshly boiled distilled water, completely inactivates the labile portion of salivary amylase immediately. Similar action can be obtained by aeration of water. The nonlabile fraction of salivary amylase is highly stable, and is not affected by gaseous oxygen.

B. Hydrogen Peroxide.—The addition of 0.1 cc. of hydrogen peroxide solution U. S. P. to 100 cc. of a 2% saliva dilution immediately destroys all amylase activity. This immediate destruction of activity indicates that the stability of the more stable fraction of amylase lies between the oxidizing power of atmospheric oxygen and that of hydrogen peroxide.

C. Bromine.—The addition of 0.05 cc. of bromine T. S. to 100 cc. of a 2% saliva dilution immediately destroys all amylase activity.

D. Hydrogen Sulfide.—Gas from the reaction of dilute sulfuric acid upon ferrous sulfide was bubbled for one minute through 100 cc. of a 2% saliva dilution. No change in activity of the salivary amylase was noted upon assay.

The Effect of Carbon Dioxide Upon the Activity of Salivary Amylase.—Since aeration has an inactivating effect upon the oxygen-labile fraction of salivary amylase, it was deemed necessary to determine whether carbon dioxide has any effect upon its activity, for it not only is a component of the atmosphere, but also plays an important role in physiological processes. Two-tenths of a gram (approximately) of dry ice was added to 100 cc. of 2% saliva dilution. Upon assay no change in the amylolytic activity was noted. Approximately the same amount of solid carbon dioxide was added to a saliva dilution which had been subjected to oxygenation. No reactivation or further inactivation occurred. It seems, therefore, that carbon dioxide in no way affects the activity of salivary amylase.

The Effect of Monosodium Glutamate Upon the Activity of Salivary Amylase.—In recent years monosodium glutamate has been popularized as a taste "activator." For years it has been used in the Orient in a crude form in the preparation of food. In the United States a relatively pure monosodium glutamate is being used to improve the taste of certain foods. An investigation was made to determine the effect of this compound upon salivary amylase activity. A solution (1%) of this com-

pound was prepared in freshly boiled and cooled distilled water. It was found to have a *p*H of 7.3. Two cubic centimeters of freshly collected saliva was diluted with 98 cc. of the monosodium glutamate solution. Two cubic centimeters of this dilution was assayed. It was found that it partially inactivated the labile portion of salivary amylase upon immediate assay. After a period of one hour it was found that the salivary amylase in this sample had been further inactivated.

The Action of Some Vitamins and Amino Acids Upon Salivary Amylase.—The importance of vitamins and of amino acids in physiological processes is well known. The following observations show the effect of the more common water-soluble vitamins and amino acids upon the activity and stability of salivary amylase.

A. Nicotinic Acid and Nicotinamide.—It was found that 2 cc. of saliva diluted with 98 cc. of a 0.0001 *M* solution of nicotinic acid in freshly boiled and cooled-to-room-temperature distilled water when immediately assayed showed almost complete inactivation of the labile fraction of salivary amylase. At the end of an hour's standing the dilution showed complete inactivation of the labile portion, with no effect, however, on the stable portion. Nicotinamide similarly assayed showed identical action. These results were duplicated in additional series of assays.

B. Ascorbic Acid.—The effect of ascorbic acid upon the activity of salivary amylase was very difficult to determine. Concentrations of ascorbic acid ranging from 0.01 *M* to 0.0001 *M* coagulated the saliva. However, by making a 0.0001 *M* ascorbic acid solution in the buffer solution and by adding 2 cc. of this buffered ascorbic acid solution in place of the regular 2 cc. of buffer solution, to the digestion mixture, one can make an assay. It was found that complete inactivation of the labile-salivary amylase occurred. Hence, an ascorbic acid concentration of 0.00002 *M* can immediately inactivate the labile-salivary amylase found in 2 cc. of 2% saliva.

C. Amino Acids.—With recently boiled and cooled distilled water, solutions were made of *d*-arginine, *l*-cystine, *dl*-tryptophane, and *l*-tyrosine. The solutions had a strength of 0.01%, except for the *l*-cystine which did not dissolve in that concentration and could be used only in a saturated solution. Two cubic centimeters of fresh saliva was diluted with 98 cc. of each amino acid solution and was assayed. Upon immediate assay it was found that all the amino acid dilutions inhibited to some degree the oxygen-labile fraction of salivary amylase. One hour after dilutions, assays were repeated, and it was found that further inhibition had occurred. The activity represented at that time was almost entirely due to the oxygen-stable amylase. Work was further continued with varying concentrations of these amino acids and inactivation was noted throughout the solubility range, from saturated solution to 0.005% concentrations.

SUMMARY

1. Protected undiluted saliva retains its maximum amylolytic activity for a period of at least one week.

2. Oxygen dissolved in water destroys a labile portion of salivary amylase. Direct aeration also destroys this labile portion.

3. Another enzyme in saliva having amylolytic activity is stable to dissolved oxygen and direct aeration.

4. Both amylolytic enzymes are destroyed by heat at 60° and by the action of a dilute solution of hydrogen peroxide.

5. The dissolved oxygen ordinarily found in water inactivates the labile fraction in fifteen minutes.

6. Storage of saliva dilutions at low temperature does not prevent dissolved oxygen from inactivating the labile portion.

7. The irregularities in the activity of salivary amylase in the same individual are not as great as the literature indicates.

8. Uniform salivary amylase activity in the same individual may be shown by one of two different methods of analysis. In one method the sample of saliva is oxygenated before the analysis is made. This gives the salivary amylase activity of the oxygen-stable form of amylase. In the other method the sample of saliva is protected against oxygen. This gives the amylase activity of both the oxygen-stable and oxygen-labile forms of salivary amylase.

9. Irregular results in the assays of salivary amylase are obtained by the ordinary methods of analysis, in which the inactivating effect of oxygen is not considered.

10. Commercial forms of pancreatin, malt extract, α-amylase, and β-amylase do not contain oxygen-labile forms of amylase. A freshly prepared pancreatin does not contain any labile amylase.

11. Nicotinic acid, nicotinamide, vitamin C, monosodium glutamate, and some amino acids all seem to have an inactivating effect upon the oxygen-labile portion of salivary amylase.

12. Continuous mastication for four hours does not affect the activity of either the oxygen-labile or oxygen-stable forms of salivary amylase. Penicillin chewing troches and sulfathiazole gum also have no effect on the activity of salivary amylase.

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Composition of Gum Turpentines of Pines. XII. A Report on *Pinus montezumae, P. oocarpa,* and *P. leiophylla**

By N. T. MIROV

Turpentines from three Mexican species of pine, *Pinus montezumae, P. oocarpa,* and *P. leiophylla*, have been investigated systematically. The physical and chemical characteristics of the turpentines studied are reported.

THIS report is the continuation of a systematic investigation of turpentines of genus *Pinus*.[1] The three pines considered in this paper are *Pinus montezumae, P. oocarpa,* and *P. leiophylla*.

These pines are very widely distributed in Mexico, and in its central part they are used as a source of commercial turpentine. Locally these pines are known as pino lacio, pino avellano, and pino chino. The oleoresin samples were collected by the author of this paper in the State of Michoacan, at the turpentining operation of the Resinera Uruapan. This operation is near the place where the Mexico City-Guadalajara highway branches to Uruapan, and is situated at 8,300 feet above sea level. Each tree from which the oleoresin was collected was carefully identified and herbarium material of each species was preserved for future reference.

EXPERIMENTAL

In Berkeley, Calif., turpentine was distilled from the oleoresin under reduced pressure, so that at the end of distillation the temperature inside the flask was 165° and the pressure was 0.5 mm. of mercury.

The characteristics of crude turpentine are shown in Table I.

The crude turpentine was fractionated with the aid of a heated-jacket distilling column of a Vigroux type, 80 cm. long. The column was equipped with a reflux still-head and a 10:1 reflux ratio was maintained during the distillation. Tails were redistilled in small flasks as indicated in Table II. The terpenes were identified by preparation of crystalline derivatives, as outlined in the previous articles of this series.[1]

Physical characteristics of the turpentine of *Pinus montezumae* show that it consists almost entirely (97%) of *d*-α-pinene; the pinene nitrosochloride melted at 103–104°. The nitrosopiperidine had a melting point of 118–119°.

Physical characteristics of *Pinus oocarpa* turpentine indicate a striking similarity of its turpentine to that of *P. montezumae*. As the oleoresin was collected from very carefully identified pines, there was no chance of confusing the two pines. It was found that 98% of the turpentine distilled below 157.75°; α-pinene was identified in this pine by the preparation of nitrosochloride, with a melting point of 103–104°, and of nitrosopiperidine, with a melting point of 118–119°.

Pinus leiophylla turpentine, although an excellent product, chemically is more complicated than the turpentine of the two other pines. Only 87% of it distilled below 157.75°; α-pinene was identified in fraction 1 by preparation of nitrosopiperidine (m. p. 118–119°); and it was concluded that about 90% of the turpentine consisted of this terpene. Attempts to identify β-pinene in fraction 7 failed, but *d*-Δ^3-carene was identified in this and in the following three fractions (8, 9, and 10) by means of preparing a nitrosate that melted at 142–143°. About 8% of the turpentine is *d*-Δ^3-carene.

Attempts to obtain a crystalline hydrochloride from fractions 16 and 17 were unsuccessful. The physical characteristics of these fractions seem to indicate that they consist of a sesquiterpene; these fractions amounted to less than 1% of the original oil.

* Received May 28, 1951, from the California Forest and Range Experiment Station, which is maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

[1] See THIS JOURNAL, **38**, 403,407(1949); **39**, 254,260(1950).

TABLE I.—CHARACTERISTICS OF CRUDE TURPENTINE

Species	Local Name	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$	Yield of Turpentine, %[a]
P. montezumae	pino lacio	0.8563	1.4655	+47.7	27.6
P. oocarpa	pino avellano	0.8569	1.4656	+47.5	24.8
P. leiophylla	pino chino	0.8515	1.4662	+23.0	29.6

[a] Based on weight of pure oleoresin (water and impurities being subtracted).

TABLE II.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS MONTEZUMAE, P. OOCARPA, AND P LEIOPHYLLA

Fractions	Boiling Range, °C.	Distillate, %	Density, d_4^{22}	Index of Refraction, n_D^{25}	Optical Rotation, $[\alpha]_D^{25}$
	Pinus montezumae (600 Gm. used; pressure 766 mm.)				
1	154.75–155.75	90.0	0.8559	1.4647	+41°00′
2	155.75–156.75	5.5	..	1.4651	+39°40′
3	156.75–157.75	1.5	..	1.4652	+37°40′
Residue above 157.75 (18 Gm.) was fractionated in a 100-cc. Claisen flask at atmospheric pressure.					
4	157.75–162.5	0.3	..	1.4678	+30°00′
5	162.5 –172.5	0.6	..	1.4680	+25°00′
6	172.5 –182.5	0.7	..	1.4757	+17°00′
7	182.5 –192.5	0.1	..	1.4764	+14°30′
8	192.5 –202.5	0.2	..	1.4815	+13°20′
9	202.5 –227.5	0.3	..	..	..
Residue above 227.5 and losses		0.8	..	..	..
	Pinus oocarpa (552 Gm. used; pressure 766 mm.)				
1	154.75–155.75	91.3	0.8559	1.4656	+42°10′
2	155.75–156.75	4.2	..	1.4659	+41°10′
3	156.75–157.75	2.3	..	1.4661	+40°00′
Residue (12.5 Gm.) was fractionated in a 100-cc. Claisen flask, at atmospheric pressure.					
4	157.75–178.0	0.4	0.8582	1.4664	+29°20′
5	178.0 –180.0	0.4	0.8595	1.4707	+26°40′
6	180.0 –183.0	0.2	..	1.4718	+25°20′
7	183.0 –203.0	0.5	0.8785	1.4754	+16°40′
8	203.0 –227.0	0.4	0.9043	1.4854	+14°40′
Residue above 227.0 and losses		0.3	..	..	..
	Pinus leiophylla (600 Gm. used; pressure 766 mm.)				
1	155.5 –156.5	79.5	0.8544	1.4660	+20°40′
2	156.5 –157.75	7.5	..	1.4668	+20°20′
3	157.75–158.5	2.2	..	1.4670	+20°00′
4	158.5 –159.5	1.7	..	1.4675	+19°20′
5	159.5 –160.5	0.8	..	1.4681	+18°20′
6	160.5 –164.5	1.0	..	1.4688	+17°40′
7	164.5 –167.5	2.6	0.8560	1.4705	+15°40′
8	167.5 –169.5	0.7	..	1.4715	+14°00′
9	169.5 –170.5	0.8	..	1.4726	+12°40′
10	170.5 –172.5	0.8	0.8566	1.4739	+12°40′
Residue above 172.5 (14.4 Gm.) was fractionated in a 100-cc. flask with a jacketed Vigroux column 8 in. long.					
11	75–80 at 20 mm.	0.2	..	1.4746	+10°00′
12	80–84	0.5	..	1.4749	+ 9°20′
13	84–90	0.3	..	1.4754	+ 8°40′
14	90–100	0.2	..	1.4740	+ 6°00′
15	100–114	0.6	..	1.4760	+11°20′
16	114–115	0.2	0.9106	1.4810	+16°40′
17	115–120	0.1	0.9513	1.4814	+24°00′
Residue above 120 and losses		0.3	..	..	..

SUMMARY

Turpentine samples of three Mexican pines were analyzed. *Pinus montezumae* and *P. oocarpa* have almost identical turpentines, consisting of 97 to 98 per cent of *d*-α-pinene. *Pinus leiophylla* turpentine consists of about 90 per cent of *d*-α-pinene, 8 per cent of *d*-Δ^3-carene, and less than 1 per cent of higher boiling fractions.

Pancreatin with High Enzymatic Activity*,†

By CHARLES W. BAUER‡ and A. JOHN VAZAKAS

A simple method of preparing pancreatin with high lipase content is outlined. An analysis, by 50 independent workers, of the pancreatin prepared by this method shows that it contains also over three times the amount of trypsin and of amylase required by the U. S. P. Thus it is an easily prepared "triple strength" pancreatin of high lipase content.

THE APPEARANCE on the market of "triple strength" pancreatin has aroused much interest in the method of its preparation. A study of several of these preparations shows that their amylase and trypsin content is three times that required for pancreatin, U. S. P. (1).

Since the literature does not give a method for preparing triple strength pancreatin in which the lipase content also is high, an investigation of this problem seemed timely. Furthermore, if such a preparation were to be used medicinally, it seemed that the effect of the stomach juices upon the lipase should be investigated.

There is given in this paper a method for making a pancreatin preparation which has a high lipase, trypsin, and amylase content. In addition, there is the evidence that shows that the acidity of the stomach irreversibly inactivates the lipase. As a result it appears that triple strength pancreatin must be enteric coated in order to get lipase activity in the intestines.

The lipase content of hog intestine was also investigated to see whether an intestinal extract that would contain more lipase than is found in pancreatin could be made from it. The results of this investigation were negative.

It appears that the pancreas is the principal source of lipase in the gastrointestinal tract and any dysfunction of the pancreas may hinder the secretion of pancreatic lipase, thus interfering with the proper digestion and absorption of fats.

HISTORICAL

In 1846, Claude Bernard (2) first observed the emulsifying action of pancreatic lipase on fat. In 1858, Marcet (3) found that lipolysis of neutral fat takes place in the stomach. Twenty-two years later, Cash (4) confirmed this finding, and also showed that extracts of the gastric mucosa could bring about fat digestion in the test tube. Ogata (5) confirmed these results. However, later authors rejected them. Volhard (6) showed that only emulsified fats are readily digested by gastric lipase.

The pancreas also contains esterases capable of splitting nonglyceryl esters, as shown by Balls and Matlack (7) in 1938 and Bauer and Masucci (8) in 1948.

In 1885, Pelouze (9) found lipases in flax seed and also in the seeds of colza, mustard, and poppy. Ricinus lipase was discovered in 1890 by Green (10) in the germinating seeds of the castor bean (*Ricinus communis*). Green found that this plant enzyme is a typical lipase, hydrolyzing true fats, similar to pancreatic lipase.

EXPERIMENTAL

Investigation of Hog Intestine for Lipase

It was suspected that the intestine might be a good source of lipase, since so much of the digestion takes place there. Although the literature states that the pancreas is the richest source of lipase, it was felt that perhaps there exists in the intestine, along with some active lipase, a lipasogen, or precursor of lipase, which could in some way be activated. A study of this problem might, it was felt, result in the finding of a new and rich source of lipase.

First, the intestine of a hog was assayed for lipase content. Any possible variation in lipase concentration throughout the length of the intestine was checked by the following method: a cleaned intestine was cut into seven pieces of approximately equal length. Each piece was numbered, beginning with number one for the lower end of the ileum, that leads into the caecum, and so on up to number seven, the proximal end of the duodenum. Each piece was also marked so that its upper and lower ends could be identified. Thus, if a concentration of lipase were to be discovered, its exact position in the intestine could be easily determined.

The method of assay of Willstätter, Waldschmidt-Leitz, and Memmen, as modified by Bauer and Wilson (11), was used.

Approximately equal-sized pieces of intestine were cut from the distal end of each of the seven portions, and each piece was weighed. They were then treated as follows:

Each piece was triturated in 50 cc. of water with some washed sand. Ten cubic centimeters of the resulting suspension was used in place of the 10 cc. of pancreatin suspension mentioned in the assay method. Four 10-cc. portions of each of the seven samples, labeled sample A, control A, sample B, and control B, were assayed. In the controls the enzyme activity was destroyed by heat (11). In all the following charts the average of sample A and sample B is given; the same is true of control A and control B. Since a preliminary assay had showed that the

* Received April 30, 1949, from the Massachusetts College of Pharmacy, Boston, Mass.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

† This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by A. John Vazakas in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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active lipase content was disappointingly low, all samples were assayed at a constant temperature of 40° instead of 30°, and for a period of two hours instead of the one hour called for in the assay, so that any marked difference in the degree of concentration would be more obvious.

The results are shown in Table I. In no case was the deviation in any duplicate set of assays greater than 0.5 cc.

TABLE I.—THE LIPOLYTIC ACTIVITY OF 8 GM. OF SAMPLE TAKEN FROM SEVEN SEGMENTS OF THE INTESTINE

Sample No.	Cc. of 0.1 *N* Alc. KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
1	18.6	18.0	0.6
2	17.8	17.2	0.6
3	17.4	16.45	0.95
4	18.3	18.0	0.3
5	19.5	18.45	1.05
6	20.1	18.25	1.85
7	19.8	18.9	0.9

An Attempt at Activation of Intestinal Lipasogen

It was thought that the pancreas might contain an activator for any lipase that was present as a lipasogen in the intestine. Consequently, a hog's pancreas and intestine were obtained, and 5 Gm. of each was assayed for lipase. The pancreas had a high degree of activity, whereas the intestine had a low degree. Then about 5 Gm. of each sample was taken. The two samples were triturated together according to the method of assay previously described, and the mixture was assayed. If any activation were to occur, the result of this assay would be higher than the sum of the results of the two separate assays. However, it was not higher, for the result of this assay was about the same as the total of the two results obtained when the pancreas and the intestine were assayed separately. Thus, even if the intestine did contain a lipasogen, it was not activated by fresh pancreas.

The results are shown in Table II. In no case was the deviation in any duplicate set of assays greater than 1.6 cc.

TABLE II.—THE AVERAGE LIPASE CONTENT OF 5 GM. OF INTESTINE, 5 GM. OF PANCREAS, AND OF A MIXTURE OF 5 GM. EACH OF INTESTINE AND PANCREAS

Sample	Cc. of 0.1 *N* Alc. KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
Pancreas	40.1	17.7	22.4
Intestine	14.9	13.9	1.0
Intestine and pancreas	39.0	18.8	20.2

Temperature and Time Suitable for Determination of Lipase Content of Hog's Pancreas

Since there was no high degree of lipase action from any portion of the intestine, and since the attempt to activate intestinal lipasogen failed, it was decided to abandon attempts to make a rich lipase preparation from the intestine; it was decided to attempt to make a pancreatin of high lipase content.

Assays were run on two pieces of fresh pancreas. One assay was run at 30° for one hour, and the other at 40° for two hours, in order to ascertain what effect an increase in temperature and in digestion time might have. The purpose of this assay was to determine at what temperature and for what period of time future assays should be run.

The average lipolytic activity, based on 10 Gm. of sample, was as follows:

At 30° for one hour..... 33.3 cc. of 0.1 *N* KOH
At 40° for two hours.... 65.0 cc. of 0.1 *N* KOH

The results above showed that there was no need to run the assays for longer periods of time than called for in the assay of Bauer and Wilson (11), or at a temperature of more than 30°, as was done in the assay of the intestines. A digestion period of one hour at 30° was sufficient to show the lipase activity of the pancreas.

The remaining portion of the assayed pancreas was then placed in toluene, for preservation. However, when it was assayed again three weeks later, a great drop in activity was noted. Evidently toluene does not preserve lipase. The result of this assay is seen in Table III.

TABLE III.—LIPOLYTIC ACTIVITY OF 10 GM. OF PANCREAS PLACED IN TOLUENE FOR THREE WEEKS, (ITS ACTIVITY WHEN FRESH HAD BEEN 33.3 CC. OF 0.1 *N* POTASSIUM HYDROXIDE)

	Cc. of 0.1 *N* Alc. KOH Required	Lipolytic Activity in Cc. of 0.1 *N* Alc. KOH	Av. Lip. Activity
Sample A	51.5	14.8	
Control A	36.7		14.9
Sample B	52.5	15.0	
Control B	37.5		

Search for a Lipase Activator

Various workers had been unable to agree whether alcohol activated or inactivated pancreatic lipase. It was decided to use various strengths of ethyl alcohol to see whether any lipase activation could be produced. Fifty per cent alcohol has been used to inhibit trypsin activity in the manufacture of insulin. Since trypsin destroys not only insulin, but also lipase, it was felt that storing the pancreas in alcohol would help obtain maximum lipolytic activity.

Determination of Lipase Content in Different Parts of the Pancreas.—In the assay just mentioned it was assumed that lipase is present uniformly throughout the pancreas. Before any further work was done, it was decided to assay a pancreas to determine whether or not it is.

A fresh pancreas of a hog was obtained and was cut transversely into four approximately equal parts. These parts were then assayed in the usual manner. The results, on a basis of a 10-Gm. sample, are shown in Table IV.

In no case was the deviation in any duplicate set of assays greater than 2.6 cc.

Table IV shows that a fairly uniform distribution of lipase existed throughout the pancreas, and that

TABLE IV.—THE LIPOLYTIC ACTIVITY OF A PANCREAS ASSAYED IN FOUR 10-GM. SEGMENTS

Segment No.	—Cc. of 0.1 *N* Alc.— KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
1	45.2	17.1	28.1
2	51.4	20.7	30.7
3	48.2	20.7	27.5
4	57.1	26.6	30.5

under that assumption, this investigation might proceed.

Some Experiments to Discover the Effect of Various Strengths of Alcohol on Lipase.—It was decided to assay some fresh pancreases of hogs; to tabulate the lipase content; and then to place these pancreases in various strengths of alcohol to see whether any particular strength of alcohol would cause activation of lipase to any marked degree. The results of the assays done to determine the lipase content of the fresh pancreases are shown in Table V.

TABLE V.—THE LIPOLYTIC ACTIVITY OF FOUR 5-GM. SAMPLES TAKEN FROM FOUR FRESH PANCREASES

Pancreas No.	—Cc. of 0.1 *N* Alc.— KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
1	22.1	15.9	6.2
2	42.0	14.9	27.1
3	36.4	15.0	21.4
4	22.1	15.2	6.9

In no case was the deviation in any duplicate set of assays greater than 4.4 cc.

When the activity of each pancreas had been recorded, the pancreases were placed in alcohol solutions of various strengths, as follows:

Pancreas 1 . . . One-half placed in 20% alcohol
One-half placed in 40% alcohol
Pancreas 2 . . . One-half placed in 60% alcohol
One-half placed in 80% alcohol
Pancreas 3 . . . Placed in undiluted alcohol
Pancreas 4 . . . Placed in 50% alcohol

Five days after the first assay the samples were assayed again for lipolytic activity. The results are shown in Table VI. In no case was the deviation in any duplicate set of assays greater than 3.8 cc.

The results in Table VI show that there was a slight increase in the lipolytic activity of the pancreas which had been placed in the 20% alcohol for the five-day period. The lipolytic activity of all the others had become less.

TABLE VI.—THE LIPOLYTIC ACTIVITY OF 5 GM. OF EACH SAMPLE FIVE DAYS AFTER IT HAD BEEN PLACED IN ALCOHOL

Sample No.	—Cc. of 0.1 *N* Alc.— KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
1—20%	23.8	15.0	8.8
1—40%	18.1	14.5	3.6
2—60%	38.2	18.8	19.4
2—80%	41.7	18.2	23.5
3—Alc.	32.8	16.4	16.4
4—50%	19.7	15.4	4.3

Eleven days after the pancreases had been placed in alcohol, assays were run again to determine the lipolytic activity of each. The results are shown in Table VII. In no case was the deviation in any duplicate set of assays greater than 3.2 cc.

TABLE VII.—THE LIPOLYTIC ACTIVITY OF 5 GM. OF EACH SAMPLE ELEVEN DAYS AFTER IT HAD BEEN PLACED IN ALCOHOL

Sample No.	—Cc. of 0.1 *N* Alc.— KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
1—20%	19.1	14.7	4.4
1—40%	18.2	15.0	3.3
2—60%	37.8	16.9	20.9
2—80%	45.2	18.5	26.7
3—Alc.	29.4	15.6	13.8
4—50%	21.8	16.1	5.7

The assays in Table VII show that after eleven days none of the samples showed any increase in lipolytic activity. In fact, all the samples showed a decrease in activity.

It is interesting to note from the figures of Tables V, VI, and VII that, although no great difference in lipase content exists in different parts of the pancreas of any one animal, a great difference does exist among the pancreases of different animals.

Preparation of a Concentrated Form of Lipase

Since all efforts to activate lipase by alcohol had failed, it was decided to proceed with the attempt to make a pancreatic preparation containing lipase in concentrated form.

The Willstätter and Waldschmidt-Leitz method (12) of making a pancreatic lipase preparation consists essentially of de-fatting the fresh pancreas by repeated washings with acetone and then of drying it with ether. The resulting dry preparation is then powdered as thoroughly as possible and is sifted. Willstätter and Waldschmidt-Leitz used this powder in their experimental work as a source of lipase. It retains its activity "for a long period of time."

This method, devised by Willstätter and Waldschmidt-Leitz, is an improvement on the method of Pottevin (13), in which alcohol and ether were used. The use of alcohol reduced the lipase content of the resulting preparation, as Willstätter and Waldschmidt-Leitz asserted, and as this investigation confirmed. Willstätter and Waldschmidt-Leitz proved this sensitivity of lipase to alcohol by preparing a sample of dried pancreas by their acetone-ether method; by then placing it in absolute alcohol for thirty minutes; by filtering with suction and drying with ether; and by then assaying it. The loss in lipolytic activity in this period of time was 14%.

The Preparation of a Rich Lipase Product by the Method of Willstätter and Waldschmidt-Leitz.—Fresh pancreases of hogs were scraped as free of fat and clinging tissue as possible. They were then put through a meat grinder four or five times, until further grinding was ineffectual. Then the glandular mass was stirred in a large mortar, 3 Kg. at a time, with a convenient amount of acetone, 1 L. at a time, until most of the fat was removed. It was then washed into large flasks with more acetone and was left to stand for several hours. The mass was then strained, and the whole process was

repeated. After the second straining, a mixture of equal parts of acetone and ether was used to wash the glandular mass. After this was done, it was washed with ether alone. By then the material was fairly gritty. It was spread upon drying paper and was dried in air. After the material was dry, it was powdered and sifted. A quantity of fibrous material remained in the sieve at the conclusion of the sifting. This material could not be pulverized. The lipolytic activity of this fibrous material was as high as that of the powdered material.

Determination of Amylase and Trypsin in the Lipase Preparation

Willstätter and Waldschmidt-Leitz had devised this method of preparing a concentrated lipase preparation so that they might have a supply of lipase constantly on hand for their experimental work. Since this method was superior to other methods of preparing pancreatin of high lipase content investigated by us, it was also of interest to determine whether this method in any way reduced the amylase and trypsin content of the pancreas. Although the method of preparation was designed to preserve as much of the lipase as possible, there is no reason why the amylase and trypsin should not be present also.

Two well-known brands of pancreatin and this new lipase preparation were assayed by the method of assay for amylase and trypsin found under "Pancreatin" in the U. S. P. XIII. It was found that the lipase preparation contained three times the amount of trypsin required by the U. S. P. and twice the U. S. P. requirement for amylase. Thus, pancreatin prepared by this method appears to be satisfactory since its amylase and trypsin content is greater than that of well-known preparations on the market today. The results of these assays are shown in Tables VIII and IX.

Although this method of making the lipase preparation is fairly easy and gives a pancreatin preparation superior to pancreatin, U. S. P., it does not seem to be in common use. The reason for this is undoubtedly that no mention has been made of its trypsin and amylase content. No reference to a commercial preparation made by this method was to be found in the literature. Although there are several triple strength pancreatin preparations on the market, the literature does not tell how they are made. Companies to whom inquiries were sent either refused to tell their method, or else referred inquiries to the United States Dispensatory (14). In the methods in the Dispensatory, alcohol, which materially reduces the lipase content of the finished product, is used.

The literature also states that glycerin may be used in the preparation of pancreatic lipase, but nowhere do the authors give the trypsin and amylase content of such a preparation. No effort was made to determine the value of glycerin in the preparing of a pancreatin of high lipase content.

TABLE IX.—AMYLASE ACTIVITY OF DIFFERENT SAMPLES OF PANCREATIN[a]

Digestion Time, Min.	No. of Assays	Color of Special Iodine Soln.		
		Sample I	Sample II	Sample III
1	2	Blue	Blue	Blue
2	2	Blue	Light blue	Blue
3	2	Blue	Colorless	Light blue
4	2	Blue	Colorless	Trace of blue
5	2	Blue	Colorless	Trace of reddish violet

[a] Sample I does not meet U. S. P. requirements for amylase content.
Sample II meets U. S. P. requirements and may be looked upon as double strength in amylase content.
Sample III barely meets U. S. P. requirements for amylase content.
These data are based upon the results of 10 independent workers.

Determination of Lipase Content of Various Pancreatins

The three samples of pancreatin having been compared for amylase and trypsin content, an assay for the lipase content was done to make the information about the three pancreatins complete. The assay was done by the usual method, and the results showed that the new pancreatin preparation had about twice as much lipolytic activity as did sample III, and about three times as much lipolytic activity as did sample I. The results are shown in Table X.

TABLE VIII.—TRYPSIN ACTIVITY OF DIFFERENT SAMPLES OF PANCREATIN[a]

Digestion Time, Min.	No. of Assays	Amount of Precipitate		
		Sample I	Sample II[b]	Sample III
10	5	+++++	+++	+++++++++
15	5	+++	++	++++++
20	5	++	−	+++
25	5	+	−	++
30	5	−	−	+
35	5	−	−	−
40	5	−	−	−
45	5	−	−	−
50	5	−	−	−
55	5	−	−	−
60	5	−	−	−
65	5	−	−	−
70	2	−	−	−

[a] Key: + means precipitation. − means absence of precipitate.
Samples I, II, and III meet U. S. P. minimum requirements for trypsin content.
Sample II is a triple strength pancreatin in trypsin content.
These results are based upon the work of 62 independent workers.
[b] Sample II is the lipase preparation made by the method of Willstätter and Waldschmidt-Leitz.

TABLE X.—THE LIPOLYTIC ACTIVITY OF 300 MG. OF PANCREATIN FROM 3 DIFFERENT PANCREATINS

Sample No.	Cc. of 0.1 *N* Alc. KOH Used by: Sample	Control	Activity in Cc. of 0.1 *N* Alc. KOH
1	27.5	17.4	10.1
2	48.8	17.4	31.4
3	37.4	20.1	17.3

The Necessity for Enteric Coatings on Lipase Preparations

A method for making a pancreatin rich in lipase having been found, the next step was to determine whether the digestive juices of the stomach would inactivate pancreatic lipase, and thus make necessary the enteric coating of lipase preparations. For this assay, a *p*H of about 1.1, which is similar to that of the stomach, was created as directed under the monograph for pepsin in the N. F. VIII. For this purpose, 35 cc. of normal hydrochloric acid was mixed with 385 cc. of distilled water.

The Method of Assay.—Three hundred milligrams of pancreatin was put into 10 cc. of the prepared hydrochloric acid, and was left standing for two hours. The acid was then exactly neutralized with 0.1 *N* sodium hydroxide and the pancreatin was assayed for lipase activity. The question was whether acidity destroys the lipase irreversibly or merely inactivates it. If the lipase is not destroyed, the neutralization of the acid should cause the lipase to be active at the *p*H of its optimum activity. If destroyed, the lipase would not be brought back upon the neutralization of the acid.

A second aim of the assay was the determination of the effect of pepsin, in the presence of hydrochloric acid, on the lipase. Since pepsin is active only in an acid medium, the two-hour digestion period at room temperature would give the pepsin a chance to destroy the lipase, if it had the power to do so.

Also, in addition to the usual controls, a control would be run in which the pancreatin would be placed, not in water, but in a solution in which 10 cc. of the acid had been neutralized by the calculated amount of alkali. This control was used to insure that no interference could be caused by the sodium chloride caused by the neutralization, which might lead to a false interpretation of the result.

The Assay.—Eight samples were prepared for analysis. The first two samples consisted of 300 mg. of pancreatin in 10 cc. of the hydrochloric acid solution. After two hours at room temperature the acid was neutralized and the preparation was assayed for lipase activity.

The next two samples consisted of 300 mg. each of pancreatin and of pepsin in 10 cc. of the hydrochloric acid solution. This mixture was left standing two hours at room temperature; then the acid was neutralized and the preparation was assayed for lipase content.

The fifth sample consisted of 300 mg. of pancreatin that was left standing in distilled water for two hours, and then assayed by the method used above. This assay was done in order to ascertain whether immersion in water for two hours would materially reduce the lipolytic activity of pancreatin.

The sixth sample consisted of 300 mg. of pancreatin assayed by the usual method for lipase content to show maximum lipolytic activity.

TABLE XI.—THE EFFECT OF HYDROCHLORIC ACID UPON THE ACTIVITY OF PANCREATIC LIPASE

Sample No.	Cc. of 0.1 *N* Alc. KOH Required
1	18.2
2	18.6
3	22.0
4	22.0
5	51.7
6	23.3
7	52.7
8	50.8

In the seventh asssay, the lipase of 300 mg. of pancreatin was destroyed by heat before the pancreatin was assayed for lipase content. Thus this assay was a control.

In the final assay, 300 mg. of pancreatin was placed in a neutralized solution of the acid to show whether the sodium chloride had any effect upon this method of determination. After the two-hour digestion period at room temperature the pancreatin was assayed in the usual manner for lipolytic activity.

Discussion.—The results in Table XI show that lipase is destroyed and not reversibly inactivated by an acidity equal to that of the stomach. Whether pepsin acts upon the lipase cannot be determined by this method, since the acid must be present in order for the pepsin to act.

SUMMARY

1. Hog's intestine contains very little, if any, lipase. If any lipase is present in the form of a precursor, it is not activated by fresh pancreas.

2. Solutions of 20, 40, 50, 60, and 80 per cent of alcohol, as well as undiluted alcohol, slowly inactivate pancreatic lipase.

3. The lipase content of a hog's pancreas is fairly uniform throughout the gland, but there is a marked difference in lipase content among pancreases of different hogs.

4. A method for the manufacture of a pancreatin of high lipase, trypsin, and amylase content is outlined.

5. Lipase is irreversibly inactivated by hydrochloric acid. It must be protected from the action of stomach juices to be therapeutically effective.

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Composition of Gum Turpentines of Pines. XIII. A Report on *Pinus albicaulis**

By A. J. HAAGEN-SMIT, T. H. WANG, and N. T. MIROV

The oleoresin of *P. albicaulis* contains 31 per cent volatile oil and 69 per cent rosin. By fractional distillation, the gum turpentine was found to contain approximately 32 per cent Δ^3-carene, 3 per cent α-terpinyl acetate, 10 per cent cadinene, 42 per cent albicaulene, 0.2 per cent albicaulol, 2.5 per cent cembrene, and about 4 per cent of residue and resinous fractions. In this unusual turpentine three compounds not previously reported as constituents of essential oils were found. *Albicaulene* is a monocyclic sesquiterpene and is related to the monocyclic tertiary sesquiterpene alcohol, *albicaulol*. The diterpene, *cembrene*, is a monocyclic diterpene.

Pinus albicaulis Engelm. (whitebark pine) is a high-altitude species found in the mountains of the Pacific coast from California and adjacent parts of Nevada to British Columbia and eastward to northern Montana and Wyoming. It belongs to the Section Haploxylon (or white pines), Subsection Cembra, Group Cembrae. To this group also belong: *Pinus cembra* of western Europe, *Pinus sibirica* of eastern Europe and Siberia, *Pinus pumila* of northeastern Asia, and *Pinus koraiensis* of Manchuria and Korea.

EXPERIMENTAL

Collection of Oleoresin.—About 3,500 Gm. of oleoresin was collected in the summer of 1946 from 20 trees growing near Mt. Rose, Washoe County, Nev., at an elevation of about 9,000 feet. The tapping started June 22 and ended August 30. Even during these summer months, air temperature was rather low, dropping occasionally at night to the freezing point. Flow of the gum was therefore exceedingly meager. Conventional turpentining methods were used and the oleoresin was collected once a week. Like all white pines so far investigated, whitebark pine has oleoresin of the consistency of honey because the rosin acids crystallize with reluctance.

Separation of Turpentine from Rosin.—The volatile oil was separated from the rosin by heating the oleoresin under vacuum until the flask residue (i. e., rosin) became hard and brittle. This point was reached when, under 0.01 mm. of pressure, the temperature inside the distilling flask reached 200°. The last 10% of distillate had the consistency of glycerol. The average yield of three distillations was as follows:

Oleoresin distilled	3,137 Gm.
Volatile oil (turpentine)	31.15 %
Rosin	68.85 %

Physical Constants of Turpentine

Density, d_4^{24}	0.8979
Index of refraction, $n_D^{20.8}$	1.4971
Specific rotation, $[\alpha]_D$	−25.1°

Fractional Distillation of Turpentine.—A sample of 650 Gm. was fractionated by distillation through a 12-in. column packed with glass helices, surrounded by a heating jacket provided with a constant reflux ratio still-head. The results are shown in Table I (see page 558).

Identification of Δ^3-Carene.—The physical constants of fractions 2 and 3 indicate the accumulation of a pure compound; these constants are summarized in Table II.

The constants (Table II) suggested the possible occurrence of Δ^3-carene and β-pinene. Failure to obtain nopinic acid by oxidation of the oil with alkaline potassium permanganate showed that β-pinene was not present. An attempt to prepare Δ^3-carene nitrosate (1) by treating a solution of the oil and amyl nitrite in acetic acid with nitric acid at 0° resulted in a good yield of the colorless crystals. After purification by recrystallization, the nitrosate melted at 146–147° and gave no depression in melting point with a sample of the known compound. (M. P. recorded in literature is 146°.)

Identification of α-Terpinyl Acetate.—Examination of Table I shows that fractions 7 and 8 contain another ingredient. Since reactions for alcohols and carbonyl compounds were negative and the elementary analysis (C, 73.5%; H, 10.2%) pointed to the presence of a terpene ester, the fraction was saponified, giving a saponification equivalent of 221.8.

The saponified liquid was distilled and a pleasant-smelling oily liquid separated out from the distillate. The aqueous residue in the distilling flask was acidified with sulfuric acid and again distilled; a volatile acid was obtained. The equivalent weight of this acid was determined by neutralizing the distillate with a measured amount of potassium hydroxide, and weighing the dry potassium salt. The equivalent weight found was 62, in close agreement with the value for acetic acid. Its presence was confirmed by the determination of the Duclaux constant (2) and the formation of ethyl acetate with alcohol and sulfuric acid.

The odor of the oily portion of the distillate strongly recalled that of α-terpineol, and the oil was identified as such by the formation of a crystalline derivative, α-terpinyl 3,5-dinitrobenzoate, which melted at 76° and gave no depression in melting point with an authentic sample of the compound.

* Received July 2, 1951, from the Kerckhoff Laboratories of Biology at the California Institute of Technology and the California Forest and Range Experiment Station, which is maintained by the Forest Service, U. S. Dept. of Agriculture, in cooperation with the University of California, Berkeley.

TABLE I.—FRACTIONAL DISTILLATION OF THE VOLATILE OIL FROM PINUS ALBICAULIS

Fraction No.	Distillation Range: Pressure, Mm.	Distillation Range: Temp., ° C.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation, $[\alpha]_D^{25}$
1	742	163–165	0.5	0.8625	1.4680	−14.78
2	742	165–167	13.7	0.8625	1.4700	−4.41
3	742	167–169	11.7			
4	742	169–171	4.7	0.8690	1.4700	+6.62
5	17	66–68	2.0			
6	17	68–70	0.4			
7	17	70–72	1.3	0.8698	1.4774	+5.29
8	17	72–75	1.0			
9	2	55–65	0.2			
10	2	65–75	0.5			
11	2	75–79	0.8	0.9218	1.4880	−14.27
12	2	79–81	0.2			
13	2	81–85	0.8			
14	2	85–92	4.8	0.9074	1.4963	−31.11
15	2	92–95	3.4	0.9131	1.4972	−35.91
16	0.01	84–108	0.9			
17	0.01	108–112	0.9		1.5080	
18	0.01	112–115	16.7	0.9186	1.5100	−94.40
19	0.01	115–120	13.7	0.9078	1.5175	−47.38
20	0.01	120–123	10.6	0.9111	1.5190	−49.54
21	0.01	123–135	1.0	0.9645	1.5270	+58.19
22	0.01	135–145	2.1			
23	0.01	145–150	0.7			
24	0.01	150–180	2.7			
Residue	...	...	4.7			

TABLE II.—IDENTIFICATION OF Δ^3-CARENE IN PINUS ALBICAULIS TURPENTINE

Properties	Δ^3-Carene[a]	Fractions 2 and 3
Boiling point, ° C.	168–171 at 1 atm.	165–169 at 740 mm.
Density	d_4^{25} 0.8668	d_4^{25} 0.8625
Refractive index	n_D^{25} 1.4675	n_D^{25} 1.4700
Molecular refraction	43.5	44.0
Carbon, %	88.23 (calcd.)	88.46
Hydrogen, %	11.77 (calcd.)	12.03
Nitrosate, m. p.	146°[b]	146–147°

[a] Dupont, G. *Ann. chim.*, **1**, 184(1924).
[b] Aschan, O., *Ann.*, **461**, 20(1928).

Identification of Cadinene.—The physical constants of fractions 14–15 indicated the presence of cadinene. This was established by preparation of the crystalline dihydrochloride. The dihydrochloride of each of the fractions melted at 117° and showed no depression in melting point with the same derivative of a known sample of cadinene.

Isolation and Study of Albicaulol, Cembrene, and Albicaulene in Fraction 18.—Upon standing for several weeks at 0°, fraction 18 yielded crystals. The crystals were filtered and separated from the last traces of mother liquor by absorption on a piece of porous porcelain plate. The crystals sublimed when heated to about 95° into beautiful, long, colorless needles which melted at 135° after purification by repeated resublimation. The total yield was 0.8 Gm.

Anal.—Calcd. for $C_{15}H_{26}O$: C, 81.08; H, 11.71; mol. wt., 222. Found: C, 81.15; H, 11.67; mol. wt., 226. One active hydrogen (Zerewitinoff method) and 1.76 double bonds.

The compound failed to react with 2,4-dinitrophenyl hydrazine and with 3,5-dinitrobenzoyl chloride. Attempts to prepare the acid phthalate derivative by refluxing a mixture of the crystalline compound and phthalic anhydride in benzene, as well as by heating a mixture of the reacting substances in an oil bath at 130° for three hours also failed. This behavior, together with the analytical data, indicated the presence of a tertiary alcohol group. None of the sesquiterpene alcohols recorded in the literature has constants corresponding to those of the alcohol described above. The name *albicaulol* is proposed for this new alcohol.

For the study of the sesquiterpenes, fraction 18 was heated with metallic potassium at 100° for several hours to remove the sesquiterpene alcohols. The reaction mixture was distilled under reduced pressure and showed the following constants:

	B. P.	d_4^{25}	n_D^{25}	M_D
Oil before treatment with potassium	112–115° at 0.1 mm.	0.9111	1.5190	67.96
Oil after treatment with potassium	180° at 20 mm.	0.9044	1.5183	68.35

Upon standing at zero degrees, the distillate solidified to a crystalline mass. After filtration, 7.5 cc. of the distillate yielded 1.4 Gm. of crystals which did not sublime as did the crystals of albicaulol obtained from this fraction before the potassium treatment. After seeding fractions 19 and 20 with these crystals, additional quantities of the crystalline material were formed. The total yield of the crystalline substance from fractions 19–20 was 15 Gm. The crystals, after being purified by recrystallization from petroleum ether, had the following constants:

Anal.—Calcd. for $C_{20}H_{32}$: C, 88.23; H, 11.77; mol. wt. 272. Found: C, 88.20; H, 11.90; mol. wt. 286. M. p. 58–59°.

Catalytic hydrogenation showed the presence of 4.1 double bonds. These data indicate that the second crystalline compound was a diterpene with one ring and four double bonds in the molecule. There are only a few diterpenes recorded in the literature, and none of them has properties identical with those of the diterpene described above. The name *cembrene* is proposed for this new diterpene.

The mother liquors from fractions 18–20, from which the two crystalline compounds had separated, and which had been distilled over potassium, consisted mainly of sesquiterpenes. Hydrogenation of these fractions indicated that they were monocyclic, containing three double bonds. Properties of these combined mother liquors are listed below:

B. P.	d_4^{25}	n_D^{25}	M_D	
180° at 20 mm.	0.9195	1.5190	67.96	found
			67.86	calcd. for $C_{15}H_{24}(=)_3$

Anal.—Calcd. for $C_{15}H_{24}$: C, 88.23; H, 11.77. Found: C, 88.56; H, 11.88. Double bonds found: 2.6.

Upon treatment with hydrochloric acid, no solid hydrochloride was formed, but a crystalline addition product was obtained with maleic anhydride which melted at 148–149°, indicating the presence of conjugated double bonds.

A search in the literature revealed no sesquiterpene with constants and other chemical properties that will correspond with those of the sesquiterpene described above. The alcohol, albicaulol ($C_{15}H_{26}O$), when dehydrated (3) by heating with fused potassium acid sulfate, gave a hydrocarbon which formed an addition product with maleic anhydride, melting at 149°, identical with that obtained from the mother liquors of fractions 18–20. Thus albicaulol and the sesquiterpene are structurally related, the sesquiterpene being the mother substance of the alcohol, and hence it will be given the name *albicaulene*. The study of the constitutions of these new compounds is in progress.

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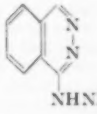
The Activities of 1-Hydrazinophthalazine (Ba-5968), a Hypotensive Agent*

By BRADFORD N. CRAVER, WALTER BARRETT, ANNE CAMERON, and FREDRICK F. YONKMAN†

The pharmacological effects of 1-hydrazinophthalazine (Ba-5968) have been reported. Its two principal actions were hypotension and partial adrenolysis. Doses of from 0.25 to 1.0 mg./Kg. intravenously usually sufficed in the dog and cat to produce the maximal hypotensive response that the drug could effect. Further marked increases in the dose exerted no obvious effect. The hypotensive action of the drug was characterized by a slow development of the maximal effect after a significant latent period. The action is probably central since the drug little influenced the blood pressure of "spinal" cats and dogs and did not affect the flow in the perfused vascular bed of the rabbit's hind limb. Both acute and chronic toxicological studies have evidenced the safety and high therapeutic index of the compound.

SEVERAL phthalazine derivatives prepared by Druey have exhibited a hypotensive activity, and among these the most active is 1-hydrazinophthalazine, (Ba-5968), which has the following structure:



Some actions of this compound have already been reported (1, 2). The compound is particularly interesting because it was shown by Reubi (3) to augment the renal blood flow concomitantly with the decline in blood pressure. This unexpected observation has been confirmed in part by Iseri, Engstrom, and Myers (4).

METHODS

All methods employed in this study have been previously reported in detail (5–7), except one. For the preparation of "spinal" cats and dogs a technique recommended by Bülbring (8) was followed. The atlas and axis were exposed from the back and their posterior bony portions removed with bone forceps. The cord was severed as high as possible, and a small segment was excised. A

* Received May 17, 1951, from the Research Department, Division of Macrobiology, Ciba Pharmaceutical Products, Inc., Summit, N. J.
† With the assistance of Elizabeth Herrold, Alfred Earl, and James Smith.

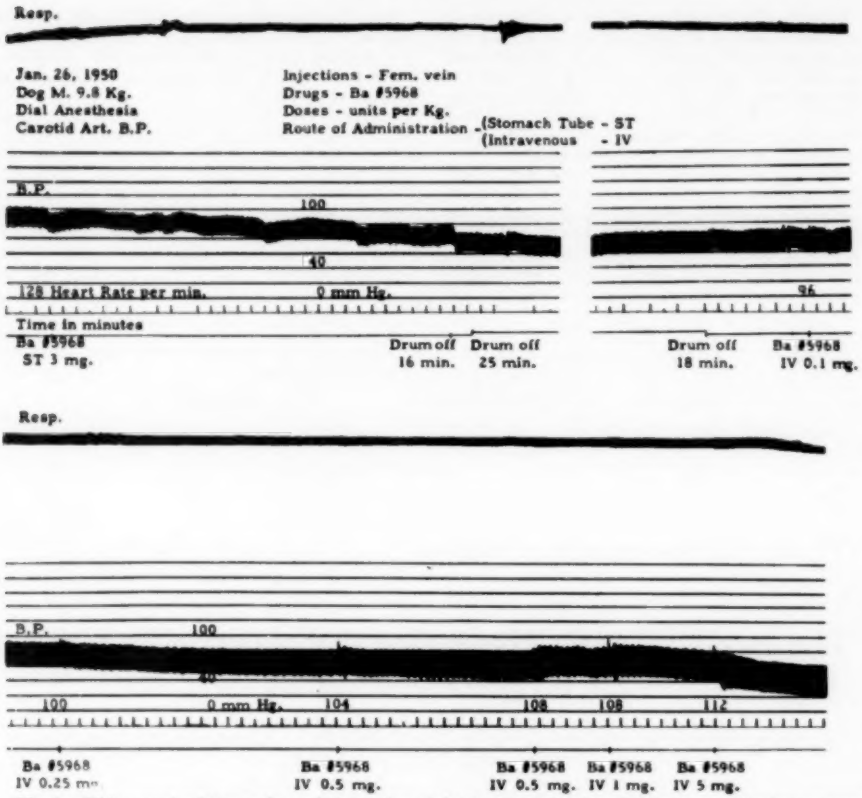


Fig. 1.—This record evidences the oral absorption of the drug and the slow development, after intravenous administration, of the drug's action. At the end of the experiment it may be seen that marked increases in the doses had but slight effect on the blood pressure.

dental probe was thrust into the brain through the foramen magnum and the brain destroyed. The probe was quickly withdrawn and a wedge-shaped piece of Plastocene stuffed into the foramen magnum to prevent hemorrhage. After the excess Plastocene had been removed, the lower cut surface of the cord was cleaned, the edges of the skin were clamped together over the wound and artificial respiration was established.

RESULTS

Isolated Tissues.—Doses as high as 100 μg./cc. exerted but a slight and inconstant spasmogenic effect upon isolated ileal strips from the guinea pig or rabbit. The drug exhibited no antihistaminic or anticholinergic action. Concentrations as high as 200 μg./cc. exerted no inhibition of the contractility of the rat-nerve diaphragm preparation. The isolated seminal vesicle of the guinea pig is contracted by both epinephrine and nor-epinephrine in doses of 1 μg./cc. Concentrations of Ba-5968 as high as 500–1,000 μg./cc. failed to eliminate entirely the response to either drug.

Effects on Intestine *in Vivo*.—Because of the slight spasmogenic action of the drug on intestinal strips, its activity was studied in dogs with Thiry-Vella loops. Doses in increments to a total of 0.6 mg./Kg. did not influence the motility of the gut. This amount was sufficient to produce a tachycardia which was taken as presumptive evidence of a hypotensive response in the anesthetized animal, and for that reason higher doses were not given.

Effects on Uterus *in Vivo*.—Doses as high as 5 mg./Kg. proved devoid of any effect on the uterus of the cat *in vivo*, although that dose sufficed to produce a maximal hypotensive response.

Effects on the Cardiovascular System.—Doses as high as 200 μg. did not influence the activity of an isolated cat heart perfused in accordance with the method of Langendorff. Doses of 100 mg. had no influence in the canine heart-lung preparation. Doses of 200 mg. usually produced slight irregularities of rhythm and sufficed to initiate a slowly progressive failure of the preparation. The drug caused hypotension in all species studied: rat, guinea pig, cat, and dog. Maximal hypotensive responses were usually elicited in all species studied by the

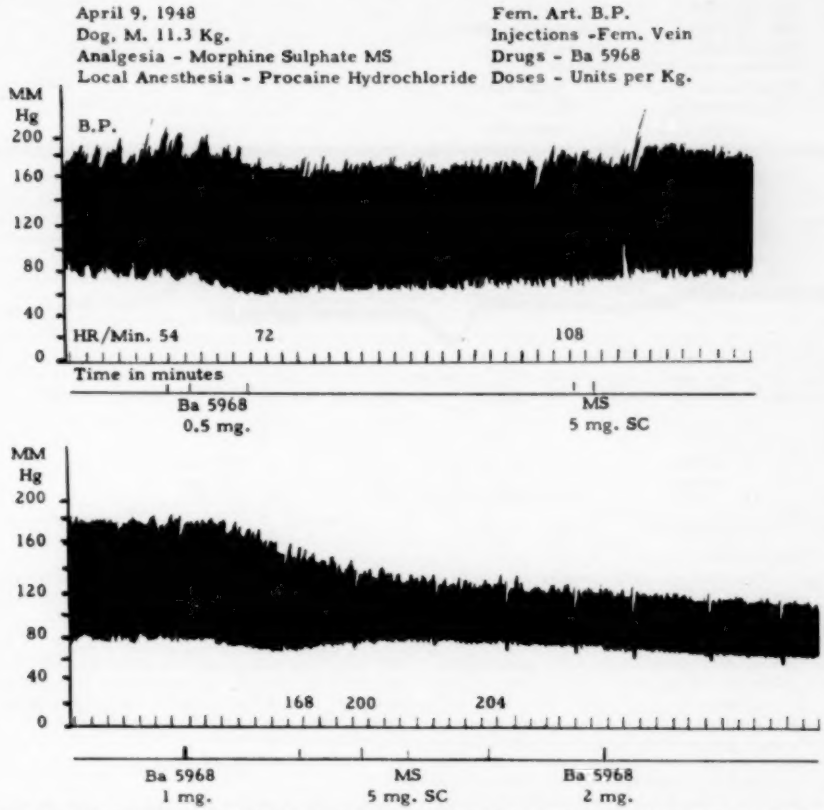


Fig. 2.—This record was obtained by arterial puncture in a dog treated only with morphine. The latent period prior to the slow attainment of the drug's maximal effect is clearly depicted. In this experiment only the systolic pressure was lowered.

intravenous administration of 0.5 to 1.0 mg./Kg. The hypotension produced by the drug had two unusual characteristics (Figs. 1 and 2): (*a*) it was slow in onset and often required several minutes after the intravenous administration of the drug before the maximal effect had been realized; and (*b*) rarely did any dose lower the blood pressure below a level of 60–80 mm. of mercury. At the point of maximal hypotension induced by the drug, histamine, amyl nitrite or acetylcholine would lower the pressure further. Ephedrine proved the most successful antagonist to the drug's hypotension, although its corrective action was at times as brief as that exerted by Pituitrin, epinephrine, and nor-epinephrine. In "spinal" dogs and cats the hypotensive action of the drug was minimal or absent. If the blood pressure of such preparations were kept at normotensive levels by a continuous infusion of 2-naphthyl-(1')-methylimidazoline hydrochloride (naphazoline), the injection of the compound gave no hypotension. Doses of 1–5 mg. did not alter the flow through the perfused hind limb of the rabbit.

Effects on Cerebrospinal Fluid Pressure.—Relatively large doses of the drug were followed either by no change in the pressure or by a slow rise (Fig. 3).

Effects on Feline Salivation and Nictitating Membrane.—Doses up to 2 mg./Kg. occasionally produced a slight reversal of the normal pressor response to epinephrine and much diminished the pressor response to nor-epinephrine. Such doses also decreased the contractile responses of the nictitating membrane of the cat to both drugs. The salivary responses to the two drugs were less frequently diminished. The salivation and contractile response of the nictitating membrane to faradization of the cervical sympathetic nerve were uninfluenced by doses in increments to a total of 5 mg./Kg. (Fig. 4).

Toxicology.—*Acute Toxicity.*—The LD_{50} for mature white rats of the Wistar strain intravenously injected with a 2% solution during a thirty-second interval was 34 mg./Kg. (method of Behrens: 63 rats were used in three groups of 10 and three groups of 11). Dogs tolerated doses of 200 mg./Kg.

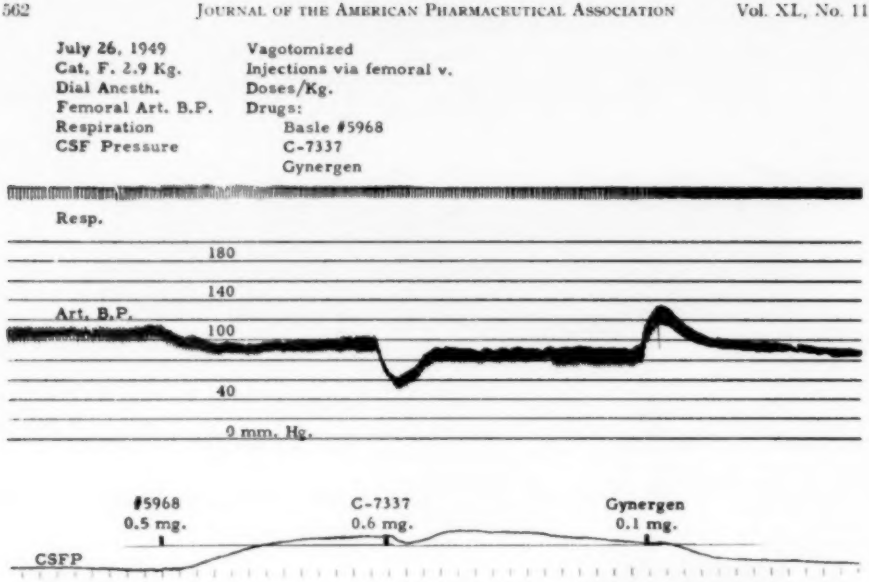


Fig. 3.—The slow rise in the cerebrospinal fluid pressure of a cat after the administration of Ba-5968 has been illustrated. The adrenergic blocking agent, C-7337 did not antagonize that effect, but Gynergen largely overcame that action and partially and rather briefly opposed its hypotensive effect.

intravenously with only tachycardia, some depression, and emesis. A dog given a dose of 300 mg./Kg. died the next day.

Chronic Toxicity.—Nine dogs, six males and three females, were fed the drug orally for an interval of three months and were divided into three groups with two males and one female in each group. At the beginning of the study a phenolsulfonphthalein test of renal function was performed on one male in each group. The hematological studies of all nine dogs included erythrocytic, leukocytic, and differential counts and determinations of hemoglobin and hematocrit. The three groups were fed respectively 10, 20, and 30 mg./Kg. orally *t.i.d.* five days a week. The animals were weighed each Monday and the drug's dosage during the following week adjusted to that weight. Cardiac rates were ascertained for each dog before the first administration of the drug and daily thereafter to determine if a cumulative action of the drug could be detected as adjudged by tachycardia. In the first two days of the experiment, cardiac rates as high as 300 per minute occurred after each administration of the doses, and accordingly the doses were changed to once a day. Despite this, the tachycardia persisted, although to a lesser degree. By Monday morning it had returned to normal. After thirty days of the drug's administration tachycardia was almost absent. One dog on a 20 mg./Kg. dose developed acute enteritis and administration of the drug was interrupted for a month. The hematological studies were repeated on the forty-fifth day of the experiment. At the end of the experiment, the weight of the animals was essentially the same as at the beginning; the test of renal clearance and all of the hematological studies were within normal limits. One dog from each group was sacrificed and tissues taken for histological examination. These tissues appeared normal at autopsy and also appeared normal after subsequent histological studies.[1]

DISCUSSION

Freis and Finnerty (9), in their recent study of the actions of Ba-5968 in man, reported that it suppressed certain sympathetic vasoconstrictor reflexes in man. This suggests man is more susceptible to the drug's possible sympatholytic action than the cat, for in the latter species no sympatholytic activity could be demonstrated. This accords also with the report of Walker, *et al.* (10). They were unable to find evidence of sympatholysis in the dog. It would indeed be unusual for a drug to cause sympatholysis that was able to produce only partial adrenolysis as evidenced not only by the drug's antagonism toward the pressor response to epinephrine, but also toward its contractile action on the isolated seminal vesicle of the guinea pig. Freis and Finnerty (9) attributed the delayed action of the drug to one of three possibilities: (*a*) it is converted to an active compound in the body; (*b*) the central mechanism it affects either responds slowly, or, (*c*) is acted upon secondarily

[1] The authors thank Dr. James Leathem for having performed the histological examinations.

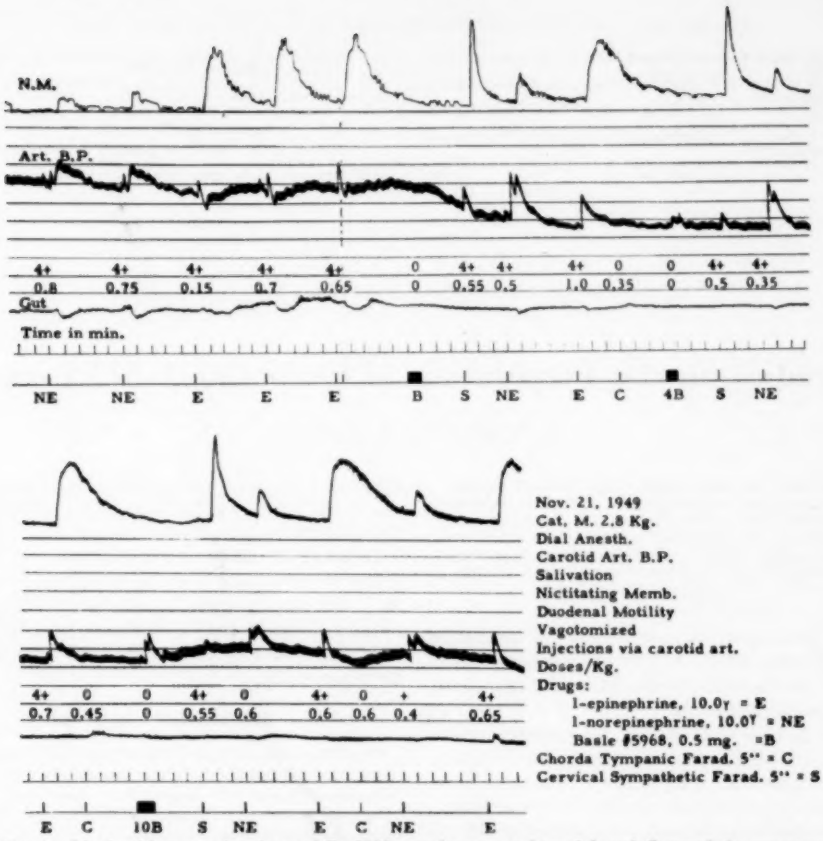

N.M.
Art. B.P.
4+ 4+ 4+ 4+ 4+ 0 4+ 4+ 4+ 0 0 4+ 4+
0.8 0.75 0.15 0.7 0.65 0 0.55 0.5 1.0 0.35 0 0.5 0.35
Gut
Time in min.
NE NE E E E B S NE E C 4B S NE
4+ 0 0 4+ 0 4+ 0 + 4+
0.7 0.45 0 0.55 0.6 0.6 0.6 0.4 0.65
E C 10B S NE E C NE E
Nov. 21, 1949
Cat, M. 2.8 Kg.
Dial Anesth.
Carotid Art. B.P.
Salivation
Nictitating Memb.
Duodenal Motility
Vagotomized
Injections via carotid art.
Doses/Kg.
Drugs:
l-epinephrine, 10.0γ = E
l-norepinephrine, 10.0γ = NE
Basle #5968, 0.5 mg. =B
Chorda Tympanic Farad. 5'' = C
Cervical Sympathetic Farad. 5'' = S

Fig. 4.—Maximal hypotensive doses of Ba-5968 may be seen to have left uninfluenced the responses of salivation and the nictitating membrane to both epinephrine and nor-epinephrine. Even the pressor responses were minimally, if at all, affected. The responses of salivation and the nictitating membrane to faradization of the cervical sympathetic nerve were not altered by the drug. The upper row of figures represents a measure of pupillary dilatation with 4+ indicating maximal dilatation. The row of figures immediately below that is a measure of salivary secretion. It represents the linear centimeters of flow along a glass tube of approximately 3 mm. internal diameter.

through an unknown chain reaction. These would appear to us also the most likely explanations; the demonstrated *in vitro* stability of the compound would not preclude the first possibility *in vivo*.

The adrenolytic action of the compound would appear to be a secondary characteristic of its action, since Ba-5968 in a dose several hundredfold the dose of 2-N-*p*-tolyl-N-(*m*-hydroxyphenyl)-aminomethyl imidazoline hydrochloride (C-7337) (Regitine) needed to eliminate the action of epinephrine on the seminal vesicle of the guinea pig, did not entirely prevent a response. Furthermore, an epinephrine "reversal" after Ba-5968 was rarely seen and then was only partial. Similar conclusions were reached by Walker and his associates (10).

The drug's focus of action is largely, if not entirely, central, since its hypotensive action in spinal animals was minimal or absent even though a normotensive level of blood pressure was maintained.

Occasional headaches have been reported in patients receiving the drug (9). The results in the experiments in which the cerebrospinal fluid pressure was recorded would suggest the headaches may be attributable to increases in the cerebral blood volume. Schroeder did not find this side-effect a serious limitation in therapeutic use (11).

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Studies on the Preparation of Quinoline-2-Aldehyde*

By VIRGINIA G. RAMSEY

A study has been made of the effect of the purity of the oxidizing agent upon the yield of quinoline-2-aldehyde obtained from the oxidation of 2-methylquinoline with selenium dioxide. Other conditions are described which result in increasing the yield of aldehyde in this reaction.

QUINOLINE-2-ALDEHYDE was an important intermediate in the preparation of compounds which were evaluated as antimalarial and antitubercular drugs.

A number of procedures for preparing quinoline-2-aldehyde may be found in the literature. α-(2-Quinoline)-β-hydroxy-γ-trichloro-propane, which was prepared readily from 2-methylquinoline and chloral by the method of Alberts and Bachman (1), was converted to β-(2-quinolyl)-acrylic acid following the work of Einhorn and Sherman (2), and this then was oxidized to quinoline-2-aldehyde according to the procedure described by Clemo and Hogarth (3). Henze (4) and Kaplan (5) reported the preparation of quinoline-2-aldehyde by the oxidation of 2-methylquinoline with selenium dioxide. All oxidation methods for the preparation of the aldehyde were accompanied by the formation of carboxylic acids and condensation by-products (3, 5, 6). In this laboratory the most direct method and the most satisfactory with respect to yield was the oxidation of the corresponding methylquinoline to quinoline-2-aldehyde with selenium dioxide. When the method of Kaplan (5) was extended to amounts of 2-methylquinoline larger than those described, the yield of aldehyde decreased appreciably. The procedure developed by Hamilton (7) for the preparation of quinoline-4-aldehyde was applied to the preparation of the 2-aldehyde, but gave poorer yields of this aldehyde than the Kaplan method.

This paper describes a study of the effect of the degree of sublimation of the selenium dioxide on the yield of quinoline-2-aldehyde. Modifications of selenium dioxide oxidation procedures which increased the yield of quinoline-2-aldehyde are reported also.

EXPERIMENTAL

The Effect of the Degree of Sublimation of Selenium Dioxide on the Yield of Quinoline-2-aldehyde.—Selenium dioxide was prepared as described in "Organic Syntheses" (8) and sublimed using the method of Hill, Soth, and Ricci (9).

In each of the tabulated runs the procedure was the same. Quinaldine (10 Gm.) was dissolved in dioxane (17 cc.) and added to a mixture of selenium dioxide (9 Gm.), dioxane (80 cc.), and water (3.2 cc.). The method followed was essentially that of Kaplan (5), only the following changes were introduced: (*a*) During the removal of dioxane from the filtrate under reduced pressure, care was taken to keep the temperature of the bath below 70°. (*b*) Before steam distillation the aqueous solution was made alkaline to phenolphthalein with sodium bicarbonate. Results of the study are summarized in Table I.

Quinoline-2-aldehyde.—The procedure for the oxidation of 2-methylquinoline to quinoline-2-aldehyde with selenium dioxide which was found to be most satisfactory is described below. Quinoline-2-aldehyde decomposed when distillation was attempted; the decomposition occurred at a bath temperature of 170° and a pressure of 2 mm.

Quinaldine (51.6 Gm.) was dissolved in dioxane (400 cc.) and heated to 60° in an oil bath on a hotplate in a flask carrying an efficient stirrer and reflux condenser. Selenium dioxide (40 Gm.), which had been freshly prepared and immediately sublimed, was added in small portions over one-half hour. The temperature during the addition rose to 88°.

* Received July 5, 1951, from the Department of Research in Organic Chemistry, Mellon Institute, Pittsburgh, Pa.

TABLE I.—THE EFFECT OF THE PURITY OF THE SELENIUM DIOXIDE ON THE YIELD OF QUINOLINE-2-ALDEHYDE

Run	Degree of Purification of SeO_2	Temperature of Reaction Mixture, °C.	Temperature of Bath, °C.	Weight of Crude Aldehyde Hydrate, Gm.	Yield, %
1	Unsublimed	95	110–115	5.0	41
2	Once sublimed	95	110–115	4.5	37
3	Once sublimed	80–85	80–85	4.6	38
4	Once sublimed then washed with 3 portions of dioxane	95	110–115	4.0	33
5	Twice sublimed	95	110–115	4.4	36
6	Residue from sublimations	95	110–115	3.2	26

TABLE II.—COMPARISON OF YIELDS OBTAINED BY VARIOUS METHODS

Method	2-Methylquinoline, Gm.	Yield of Crude Quinoline-2-aldehyde, %	Yield of Purified Quinoline-2-aldehyde, %	Melting Point, °C.
Hamilton (7)	80.0	..	11	67–69
Kaplan (5)	77.0	19	..	..
Modified	51.6	30	22	67–69
Modified	59.3	30	20	67–68

After the addition of all the selenium dioxide the reaction was continued at a temperature of 75–88° for one-half hour and then the solution was filtered hot to remove the precipitated selenium. Dioxane was removed under reduced pressure at a temperature which was maintained below 70°. Water (400 cc.) was added to the residue which remained after evaporation of the solvent. The solution was made alkaline to litmus with sodium bicarbonate and steam distilled. The distillate was chilled overnight in a refrigerator before filtering off the quinoline-2-aldehyde hydrate.

On drying the hydrate in a desiccator over phosphorus pentoxide, 17.0 Gm. of crude quinoline-2-aldehyde was obtained; yield, 30%. This material was crystallized from Skellysolve (3 cc. of Skellysolve to 1 Gm. of aldehyde) and dried to constant weight in a desiccator over phosphorus pentoxide and paraffin. The crystallized quinoline-2-aldehyde was found to weigh 12.5 Gm. and melt at 67–69°; yield, 22%.

A comparison of the yields obtained using the different selenium dioxide oxidation procedures is given in Table II. In all cases the selenium dioxide had been freshly prepared and once sublimed.

SUMMARY

1. The effect is shown of the purity of the selenium dioxide upon the yield of quinoline-2-aldehyde for the reaction in which quinoline-2-aldehyde is prepared by the oxidation of 2-methylquinoline with selenium dioxide.

2. Conditions are described for the preparation of quinoline-2-aldehyde, from 2-methylquinoline by oxidation with selenium dioxide, in improved yields.

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American Association for the Advancement of Science—Pharmacy Subsection Meeting

The next meeting of the American Association for the Advancement of Science will be held in Philadelphia on December 26–31, 1951. The program of the Pharmacy Subsection of the Medical Sciences has been made up. Six sessions of this Subsection will be held. Members are invited to attend the meetings of the Section on Medical Sciences, Subsection on Medicine, and the Section on Chemistry.

Study of Thiochrome Solutions under Various Lighting Conditions*,†

By LEON J. DeMERRE and MARY ANN SEIBOLD‡

The rate of destruction of thiochrome solutions, obtained from thiamine hydrochloride, when exposed to direct sunlight, shade, dim daylight, artificial light, and various monochromatic light sources has been determined. Direct sunlight, light above 600 foot-candles from an incandescent bulb, ultraviolet light, and monochromatic light sources in the short wave-length region all cause destruction of thiochrome solutions in a short time. Dim daylight not higher than 10 foot-candles is not detrimental to thiochrome for a reasonable length of time. Similarly, light from monochromatic sources in the green region (485 mμ) and above causes no destruction.

THE TEXTBOOKS and papers describing the thiochrome method for the fluorometric determination of thiamine frequently issue a warning against exposure to ultraviolet light and bright daylight (1) or merely state that the material is unstable to light. Recommendations to the effect that measurements should be performed rapidly and in a semidark room usually follow (2).

To our knowledge no systematic study of the effect of various lighting conditions on thiochrome solutions has been published. The great number of fluorometric assays of thiamine requested from pharmaceutical laboratories prompted a study of the conditions of illumination under which reliable determinations of thiamine could be made.

The present work deals with the rate of destruction of thiochrome solutions, obtained from pure thiamine hydrochloride,[1] exposed to direct sunlight, shade, dim daylight, artificial light, and various monochromatic light sources. Our work was performed on isobutyl alcohol solutions of thiochrome derived from buffered aqueous solutions of thiamine hydrochloride in the concentrations of 1.5, 1.0, 0.75, 0.5, and 0.25 micrograms per milliliter.

EXPERIMENTAL

General Procedure

A stock solution of thiamine hydrochloride in 0.01 *N* hydrochloric acid containing 100 μg./ml. was prepared. Dilutions were made from this stock solution to the above-mentioned concentrations, by using standard techniques (1, 3, 4).

Technique of Exposure

Ten-milliliter quantities of the solutions were placed in 50-ml. Pyrex beakers. This brought the depth of the solutions to about 1 cm.

An original sample of each concentration was taken and corresponds to the highest point—or zero time point—on the individual destruction curve. Additional samples of all these solutions were taken after fifteen, thirty, sixty, and one hundred and twenty minutes of irradiation, air being the only medium between the light source and the solution. All the samples for a complete series of experiments were stored in a box, insuring complete darkness. They were measured fluorometrically at the conclusion of the last exposure. In every case the light was allowed to strike the surface of the solution perpendicularly. It was observed that the possible destruction of thiochrome that may occur during the short time of exposure to the ultraviolet source of the fluorometer necessary to make a reading is negligible.

Equipment and Preliminary Experiments

The Coleman Universal Spectrophotometer Model 11 with the Ultraviolet Illuminator Attachment Model 14-030 was used for all readings.

The preparation of the solutions, their subsequent manipulations, and all fluorometric readings were in nearly total darkness.

Preliminary experiments showed that in concentrations above 1.5 μg./ml. a white cloudiness may appear. Therefore 1.5 μg./ml. was the highest concentration used.

The slight fluctuations in the current or in the apparatus itself were negligible.

Lighting Experiments

A. Exposure to Natural Light (Fig. 1).—This includes three intensities which are summarized in Table I.

TABLE I

Nature of Light	Light Intensity in Foot-Candles[a]
Direct sunlight	10,000
Daylight in shade	500
Dim daylight	10

[a] A Norwood Director Exposure meter, American Bolex Co., was used.

* Received May 29, 1951, from Biophysics Laboratories, Inc., Los Angeles, Calif.
† This work was supported by a grant from E. P. Garnier.
‡ The authors are grateful to Dr. L. Zechmeister for his views on the chemical aspects of this study, and to Mr. R. W. Benoliel and Dr. S. Rubin regarding the physics involved in the experiments.
[1] Thiamine hydrochloride U. S. P. for Ampuls, Merck and Co., Inc.

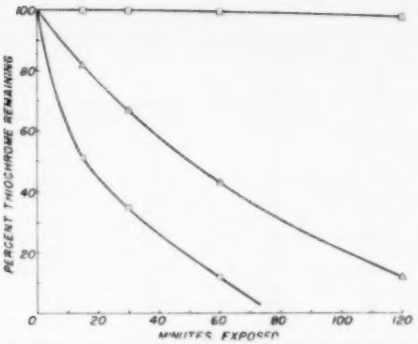

Fig. 1.—Natural daylight. ⊡ Dim daylight, 10 foot-candles; △ shade, 500 foot-candles; ⊙ sunlight, 10,000 foot-candles.

1. Direct Sunlight.—The brightness of the sunlight was practically constant. All sunlight exposures were made between 11 a. m. and 1 p. m. in order to avoid any significant changes. Destruction takes place immediately and at a rapid rate. Total destruction occurs after seventy to eighty minutes of exposure.

2. Daylight in the Shade.—The beakers were arranged in a location where an intensity of 500 foot-candles could be assured for the duration of the experiments. In all cases studied, a sharp destruction occurred. Proportionally considered, the destruction was greater than with direct sunlight. This seems to indicate that the brightness is not only the factor involved, but that the quality of the light is also important.

3. Dim Daylight.—The brightness for these experiments was adjusted to 10 foot-candles. This intensity of illumination permits one to extend appreciably the working period without noticeable eyestrain (5). In no case was destruction observed. The readings remained steady within the limits of error.

b. Artificial Light (Fig. 2).—A 150-watt reflector-type filament lamp was used and the average voltage was 112. Variations of the light intensity were obtained by changing the distance between the light source and the solution (see Table II).

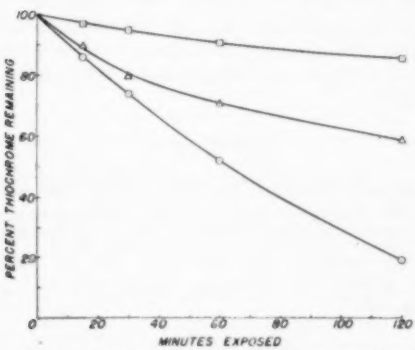

Fig. 2.—Artificial light. ⊡ 100 cm., 600 foot-candles; △ 50 cm., 1400 foot-candles; ⊙ 25 cm., 2800 foot-candles.

TABLE II

Distance between Light Source and Solutions, Cm.	Light Intensity in Foot-Candles
25	2,800
50	1,400
100	600

In each case the same concentrations of thiochrome were used. The beakers were placed on a dark-gray background to avoid reflection. The combined curves clearly show a greater destruction as the light intensity increases.

C. Ultraviolet Light (Fig. 3).—A Westinghouse Sterilamp in which 80% of the radiations generated are in the region of 2537 Å. was employed in this study. The tube was 30 in. long and totaled eight hours of service before the experiments. Since an appreciable loss of efficiency is not noticeable before 4,500 hours of service, it is safe to assume that the successive experiments reported here were performed under the same conditions.

Although no factor of proportionality could be established, the curves clearly show that the destruction of thiochrome increases as the distance between the light source and the solutions diminishes. Destruction occurs at an appreciable rate from the very beginning. This implies that fluorometric readings, to be accurate, must be made rapidly.

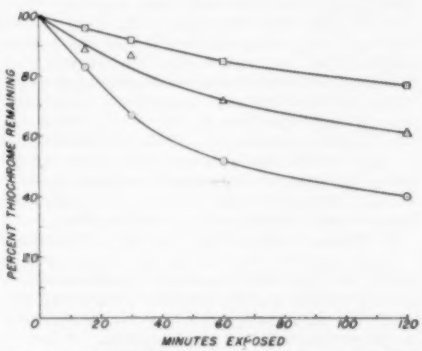

Fig. 3.—Ultraviolet light. ⊡ 100 cm., △ 50 cm., ⊙ 25 cm.

D. Quasi-Monochromatic Light Sources.—The same 150-watt reflector lamp was used in this series of experiments in conjunction with Corning glass color filters. The maximum transmission wave lengths of the filters used were: violet-blue (365 mμ); blue (395 mμ); blue-green (485 mμ); green (515 mμ); and deep red (610 mμ). The illumination with each filter was studied at two distances, 25 and 50 cm., between the surface of the solution and the light source (Fig. 4). The necessary precautions were taken to exclude stray light from all experiments.

The brightness of these various quasi-monochromatic light sources cannot be given in absolute

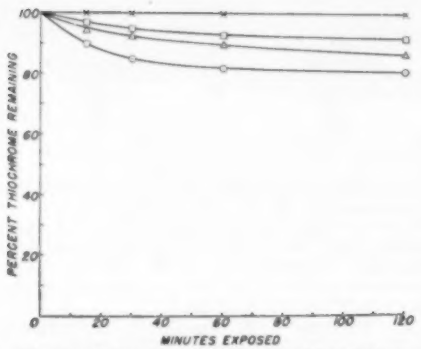

Fig. 4.—Colored filters. X Green 25 and 50 cm.; blue-green 50 cm.; red 25 and 50 cm. ⊡ Blue-green 25 cm. △ Violet-blue 50 cm.; blue 25 and 50 cm. ⊙ Violet-blue 25 cm.

values. In order to present a means of reproducing the exact conditions of the work and to have numerical values for comparative purposes, Table III was established.

The exposure meter was used to measure the brightness of the artificial light, first without any filters at the two above-mentioned distances, and then with filtered lights. The curve of relative response of the exposure meter[3] to the various wave lengths used gives an idea of what the actual brightness should be. The latter is normalized to the peak transmission of the particular wave lengths.

1. Exposure to Violet-Blue Light (365 mμ).—The curve of average destruction for the various concentrations used shows that for a distance of 25 cm. the greatest destruction occurs after thirty minutes of exposure and amounts to some 15%. This slowing-down process, which is also observed with the other color filters, may be explained by the fact that on the solutions, which are never stirred during the entire time of exposure, there may have developed a protective layer of altered material. At twice the distance half the destruction occurs.

2. Exposure to Blue Light (395 mμ).—The curves for both distances—except for the 25-cm. distance during the first half hour—are superimposed on those of the 365 mμ experiments. The 30 mμ difference between the maximum transmission of the two filters did not prove discriminatory.

3. Exposure to Blue-Green Light (485 mμ).—For a distance of 25 cm. a very slight destruction occurred, amounting to as little as 8% after two hours of exposure. At a distance of 50 cm. from the light source the strength of the solution remainted intact.

4. Exposure to Green Light (515 mμ).—No destruction was observed, for both distances.

5. Exposure to Red Light (610 mμ).—In no case was destruction observed.

[3] Curve furnished by the meter manufacturer.

TABLE III

Characteristics of Colored Filters—Maximal Transmission in mμ	Distance from Light Source to Surface of Soln., Cm.	Brightness in Foot-Candles with Filter— Relative Response	Normalized Response[a]
365	25	120	218
	50	65	118
395	25	110	157
	50	56	80
485	25	156	174
	50	80	89
515	25	200	206
	50	110	113
610	25	190	210
	50	100	111

[a] Without filters the brightness of the lamp is 2,800 foot-candles at 25 cm. and 1,400 foot-candles at 50 cm.

CONCLUSIONS AND SUMMARY

1. Direct sunlight produces total destruction of thiochrome solutions in a very short time. Natural daylight also produces total destruction. As anticipated, the rate of destruction is slower than in the case of direct sunlight, but not substantially.

2. Dim daylight in intensities not higher than 10 foot-candles is not detrimental, for a reasonable length of time, to thiochrome solutions. This brightness is sufficient for working purposes and thiochrome assays may be made under those conditions.

3. Light from an incandescent bulb has a marked destructive effect for brightnesses above 600 foot-candles.

4. Ultraviolet light acts similarly to sunlight. A quantitative relationship is not presented here since comparative brightness is difficult to estimate.

5. For monochromatic light sources, whose intensities are in the same range, destruction is more efficient in the short wave-length region. In the green region (485 mμ) and above no destruction occurs. The intensities of the green and red lights used are ample for working conditions.

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The Effects of Freezing Temperature Storage on Digitalis Leaf Glycoside Yields*,†

By H. W. YOUNGKEN, Jr., E. H. DJAO, and D. P. N. TSAO‡

Fresh digitalis leaf material has been subjected to freezing temperature storage for a two-year period and compared in glycoside content with dried material from the same growth. Higher glycoside yields were obtained following freezing temperature storage. Advantages and disadvantages of the process are suggested.

THE conventional methods for preparing digitalis tinctures or for the isolation and purification of the active glycosides make use of dried leaves as starting material. In general, following the collection of fresh leaves, these are subjected to rapid drying with artificial heat and at temperatures from 85 to 150° F. Opinions vary considerably as to the desirability for rapid drying at very high temperatures, for example, 120° F. or more. Koch and Butler (1) have recommended initial heating at 100° F. for one hour and then rapid drying at much higher temperatures. Hale (2), Haskell and Miller (3) and others have used temperatures of 120° F. and found no deleterious effects. However, they noted that temperatures higher than 120° F. generally resulted in a loss of glycoside activity. On the other hand, Hughes and Todd (4) and Wijngaarden (5, 6) have shown that continuous artificial drying at about 60–150° F. generally was a satisfactory range for optimum glycoside yields and that initial rapid drying at higher temperatures was unnecessary. This broad temperature range is commonly employed for leaf drying procedures by drug plant collectors.

The principle observed during the preparation of digitalis leaves for their extraction is, of course, the inhibition of those enzymes present which are known to effect hydrolysis of the active glycosides. Stoll and Kreis (7) have shown rather conclusively the importance of inactivating digitalis enzymes prior to glycoside extraction. Rapid drying under temperature conditions described above does inhibit enzyme function in digitalis. It is also common knowledge that freezing temperatures will inhibit enzyme functions. Hepler, *et al.* (8), have measured the potency of digitalis plants which were frozen in the field and found a high degree of activity despite their brownish color. However, the effect of a long period of freezing temperature storage on digitalis leaf glycoside yields has not been conclusively determined. It would seem practical at times for leaf collectors to employ freezing temperature storage when fresh material is available and dryer space or facilities are not.

The objectives of this study have been then, (*a*) to determine the effects of freezing temperatures on digitalis leaf glycoside content following a long period of leaf storage under freezing conditions, and (*b*) to compare these results with total glycoside yields of leaf material from the same source which has been rapidly dried.

EXPERIMENTAL

Digitalis leaves were collected during August, 1948, from each of five experimental plots of first-year plants growing in the Drug Plant Gardens, University of Washington. Plants had been developed from a parent strain of mixed color *Digitalis purpurea*, and all were of the same age when collected. Aliquots of leaf material from each plot were separated into two groups and weighed. One group was set aside for immediate drying and the second was placed at once into two kinds of containers for freezing. One container consisted of a conventional heavy-duty ice cream "Jiffy Bag"[1] whereas the other was an ordinary thin brown 10 in. x 14-in. Manila envelope. These containers were placed into an 8-cu. ft. freezing compartment in which leaf material was then stored for a period of twenty-four months at a temperature of from 0 to 10° F.

Leaf material of group I, which was not frozen prior to examination, was rapidly dried in a current of forced circulating air at temperatures of from 85 to 100° F., (29–38° C.) in a laboratory drug dryer. From five to six days were required for thorough drying. Dried material was then weighed and ground to a No. 40 powder and tinctures were made from this material according to the U. S. P. XIII method (9). These tinctures were then placed into several 50-cc. ampuls in which they were stored in an atmosphere of nitrogen. Each tincture was assayed for total digitalis glycoside content by the Bell and Krantz (10) procedure for digitalis tincture. Colorimetric determinations were made with the use of a Beckman DU Spectrophotometer during which 1-cm. cells were employed and readings were made at 525 mμ. Readings were compared with determinations similarly made of U. S. P. Digitalis Refer-

* Received July 5, 1951, from the College of Pharmacy, University of Washington, Seattle.
† Aided in part by a grant from the State of Washington Fund for Medical and Biological Research.
‡ Drug Plant Laboratories, College of Pharmacy, University of Washington, Seattle.

[1] Jiffy Manufacturing Company, Hillside, N. J.

TABLE I.—DIGITALIS GLYCOSIDE DETERMINATIONS: DRIED LEAVES COMPARED WITH FROZEN LEAVES[a]

Lot No.	Dried Leaves: No. of Assays	Dried Leaves: % Potency in Terms of U.S.P. Ref. Std.	Frozen Leaves: No. of Assays	Frozen Leaves: % Potency in Terms of U.S.P. Ref. Std.	Difference, %
1	3	95	4	128	33.5
2	4	60	4	81	31.5
3	3	65	4	85	30.5
4	4	104	4[b]	113	8.0
5	4	117	4[b]	143	24.0

[a] All assays were made according to the Bell and Krantz (10) colorimetric glycoside analysis method for digitalis tincture.
[b] Lot Nos. 4 and 5, leaf material stored in heavy-duty freezing bags; all other samples stored in thin Manila envelopes for freezing purposes.

ence Standard. Results of these glycoside assays are shown in Table I.

In order to compare the effects of freezing temperatures (0 to 10° F.) on leaf storage and its glycoside content, samples of group II were removed from the freezing compartment approximately two years later. All leaf material was found to have an excellent green appearance and leaves had dried considerably as the result of this storage. Leaf samples that had been stored in ordinary Manila brown envelopes were found to have dried more so than those kept in heavy-duty "Jiffy Bag" containers. For example, the average moisture content from several hundred grams of fresh weight stored in thin Manila envelopes was about 35%; whereas that from similar quantities of fresh weight material stored in heavy-duty bags was about 60%. It was evident (see Table II) that the moisture content in both cases of frozen leaves stored for two years was higher than the 6% maximum for U. S. P. digitalis.

To a certain extent the per cent of moisture found in dried digitalis is a criterion of thoroughness of drying and glycoside deterioration. However, although originally it was thought that the amount of moisture allowed in dried drug was a critical factor within a narrow range, such as below 2%, the reports of Haskell and Miller (3), Rowe (11), Christensen and Smith (12) and others indicate that deterioration due to moisture during room temperature storage is not significant below 6%. Furthermore, many of these reports indicate that air-tight and light-tight containers are not entirely necessary for proper storage. The Pharmacopeia does limit the moisture in digitalis U. S. P. to 6%, although no restrictions are placed upon material for glycoside extraction. However, in testing digitalis leaves from samples of frozen material for glycoside content the large moisture content must be taken into account when tinctures of this material are to be compared with those of dried samples. Therefore, during the preparation of digitalis tinctures from frozen leaves, a modification of the U. S. P. method was made. This involved an adjustment of the water content of the 4:1 alcohol-water mixture which is specified for each 10 cc. of solvent per gram of dried powder. Aliquots of frozen leaf material were set aside for moisture content calculations. After determining the moisture content of these by the official method, the moisture content of nondried samples was calculated. Thereupon tinctures of frozen leaves were prepared by using, per gram of calculated dry weight, eight parts of alcohol and two parts of water, minus the amount of calculated water in the sample. Proper calculations for moisture content are necessary at this point and this factor is, perhaps, a disadvantage that frozen leaf processing entails, for each sample must be examined for its moisture content before tinctures can be prepared.

Frozen samples were disintegrated in a Waring blendor for ten minutes with most of the menstruum, which was made by mixing correct proportions of alcohol and water as stated above. The mixture was then transferred to a glass-stoppered flask; the remainder of the menstruum was used to rinse any residue from the Waring blendor into the collecting flask. All mixtures were shaken for twenty-four hours. They were then centrifuged, filtered, and placed into 50-cc. ampuls under nitrogen. The Bell and Krantz (10) colorimetric assay procedure was employed for glycoside analysis in the same manner as for material from the dried source. Results of analyses of different samples of digitalis frozen leaf material are shown in Table I. It was observed that glycoside contents were consistently higher in samples which had been frozen for the two-year period than in samples of the same source of leaf drug which had been dried immediately after harvest. For example, the glycoside yields in

TABLE II.—MOISTURE DETERMINATIONS: DIGITALIS LEAVES FROZEN FOR 2-YEAR PERIOD

Lot No.	Fresh Wt., Gm.	% Moisture Content Fresh Wt.	Wt. After 2-Yr. Freezing Temp. Storage, Gm.	% Moisture Content After Freezing Storage
1[a]	180	86.00	42	40.0
2	200	85.24	48	38.5
3	350	81.44	87	25.4
4	450	85.34	104	36.6
				Av. 35.1
5[b]	300	86.5	190	78.7
6	109.5	85.0	31	47.0
				Av. 62.3

[a] Leaves frozen in 9 x 12 in. Manila envelopes (thin).
[b] Leaves frozen in heavy-duty "Jiffy Bag" containers.

TABLE III.—COMPARATIVE GLYCOSIDE YIELDS OF DIGITALIS LEAVES DRIED AT LOW AND HIGH TEMPERATURES[a]

Drying Temp., ° F.	Sample Fresh Wt., Gm.	Dry Wt., Gm.	Tr. No.	No. of Assays	% Potency in Terms of U. S. P. Ref. Std.	Average % Potency
85–100	600	110	1	3	96	97
			2	3	97	
60–75	600	112	3	3	101	100
			4	3	101	
			5	3	99	

[a] Glycoside determinations according to Bell and Krantz colorimetric method for digitalis tincture.

frozen samples measured from 8 to 33% higher (see Table I) and most samples of this material assayed more than 20% higher in glycoside than those which were prepared from dried leaves. Two samples from an experimental plot of digitalis leaves which were known to assay low in total glycoside content when dried also showed a corresponding higher yield (about 30%) when stored for two years at a freezing temperature.

There appeared to be no significant differences between total glycoside contents in frozen leaf material which had been stored in thin envelope containers when compared with that kept in heavy-duty "Jiffy Bags." From the standpoint of moisture content and subsequent calculations of this factor for the preparation of tinctures it was concluded that thin bag-type containers would be more desirable than others for freezing temperature storage. These bags are frequently less expensive than the heavy-duty type.

In order to test the possibility that some damage to glycoside content might have taken place during the forced hot-air drying at temperatures from 85 to 100° F., it was deemed important to repeat this phase of the experiment. Several additional leaf collections were made, therefore, from one-year digitalis plants and aliquots of these were dried at temperatures of two ranges. One range was that employed previously, i.e., 85 to 100° F.; the second was a circulating room temperature of from 60 to 75° F. All material was dried thoroughly for glycoside analysis. Samples from both groups were then powdered (No. 40) and tinctures were prepared and examined for digitalis glycosides. No significant differences were observed between the glycoside yields of the two groups (see Table III). From these data it was concluded that glycoside yields in leaf material dried between a wide range of temperatures (60–100° F.) were about the same. On the other hand, since yields were considerably greater in frozen material of the same source (Table I), some loss in total glycoside must be assumed to take place during the conventional drying process.

Subsequent Drying of Frozen Material.—In view of the differences between glycoside yields of frozen and dried leaf materials, several aliquots of two-year frozen digitalis were placed into the drug dryer and dried for one week at a temperature of 85 to 100° F. Aliquots of frozen material of the same source were immediately prepared for glycoside comparisons. Samples were then assayed for total glycosides in the same manner as previously described for powdered and frozen digitalis. The results are shown in Table IV. It was found that all samples of dried leaf material but one gave lower yields of glycoside. Although the decrease in amount was not as great as formerly observed when nonfrozen leaf material was tested, nevertheless there was sufficient evidence by it to indicate that drying at this temperature causes some loss in digitalis glycoside yields. The loss appeared to be less when samples were previously frozen before drying.

Advantages and Disadvantages of the Method.—Advantages and disadvantages were noted during the freezing storage process. Higher yields of total glycosides were found in each analysis of frozen leaves. Leaf material generally appeared greener and in other aspects better than did dried leaves. In several cases when frozen leaves were subsequently dried they retained their green color more so than did those dried immediately after harvesting. Furthermore, there was less loss of glycoside when this material was subsequently dried.

From time to time digitalis leaf collectors seek means for temporarily storing fresh leaves when drug dryers are not immediately available. On the basis of the results shown during these experiments it is suggested that ordinary freezing lockers in which temperatures are generally kept at 0–10° F. can be utilized for this purpose.

The major disadvantages noted during the freezing temperature process were the following: The

TABLE IV.—DIGITALIS GLYCOSIDE DETERMINATIONS: FROZEN LEAVES COMPARED WITH FROZEN LEAVES SUBSEQUENTLY DRIED[a]

	Frozen Leaves			Frozen Leaves Subsequently Dried			
Lot No.	Wt., Gm.	No. of Assays	% Potency in Terms of U. S. P. Ref. Std.	Wt., Gm.[b]	No. of Assays	% Potency in Terms of U. S. P. Ref. Std.	% Difference
1	25	4	92	15.14	4	82	12
2	25	4	76	16.39	4	72	7
3	25	3	96	17.95	3	88	9
4	25	3	98	16.23	3	104	−6

[a] All analyses were made according to the Bell and Krantz (10) colorimetric glycoside assay method for digitalis tincture.
[b] When dried from 25-Gm. aliquots of frozen sample.

time required for preparing standard tinctures from such material is lengthy, although the same quantities of alcohol solvent are employed. The separation of these tinctures requires careful determination of the moisture content which is a variable factor, in frozen leaf material. Furthermore, more steps are necessary during the method than generally encountered during the preparation of digitalis tinctures from dried and powdered crude drug, i. e., disintegration in a Waring blendor and the selection of proper menstruum proportions. Frozen material requires larger space for storage and obviously cannot be transported at the same economic rates as can dried leaf drug.

SUMMARY AND CONCLUSIONS

A study has been made of the effects of freezing temperature storage for a two-year period on total glycoside content in *Digitalis purpurea* leaves. Results indicate that fresh leaf material can be kept under these conditions without loss in total glycosides as assayed by a colorimetric method.

There was evidence that the glycoside content of leaf material stored under freezing conditions as herein described was higher than unfrozen material of the same source which had been dried.

It is suggested that some loss in glycoside yields must take place during the conventional drying process for digitalis leaves.

Advantages and disadvantages are suggested for the comparative freezing temperature storage and drying processes.

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Infrared Analysis of Pharmaceutical Products Containing Sodium Propylmethylcarbinylallyl Barbiturate and Sodium Isoamylethyl Barbiturate*

By A. M. RIBLEY, E. E. KENNEDY, W. W. HILTY, and T. V. PARKE

A rapid control procedure is given for analysis by infrared absorption of pharmaceutical products containing sodium propylmethylcarbinylallyl barbiturate and sodium isoamylethyl barbiturate by which the individual components may be determined simultaneously and with only moderate mutual interference.

AT THE present time chemical separation of sodium propylmethylcarbinylallyl barbiturate and sodium isoamylethyl barbiturate is virtually if not entirely impossible. Therefore, the usual methods of assay have amounted to a determination of total barbituric acids by gravimetric, titrimetric, or ultraviolet absorption techniques. A method utilizing infrared absorption has been developed by which the individual components of pharmaceutical products containing sodium propylmethylcarbinylallyl barbiturate and sodium isoamylethyl barbiturate may be simultaneously determined. It was the concern of these laboratories to develop a method for assay of products containing the sodium salts; however, the method developed applies equally well to products containing the free barbituric acids.

* Received July 9, 1951, from Eli Lilly and Company, Indianapolis, Ind.

EXPERIMENTAL

Spectra have been obtained with a Baird Infrared Recording Spectrophotometer Model A equipped with rock salt prism. The slit mechanism of this instrument has been altered to give slightly wider slits than originally provided. Repeatability in per cent transmission recording is about ±0.2% and the

resolution is slightly reduced. The cell path lengths are measured by the interference method (1) or by direct measurement with a microscope. No attempt was made to match precisely the sample and the reference cells.

Infrared absorption spectra for propylmethylcarbinylallyl barbituric acid, isoamylethyl barbituric acid, and a 1:1 mixture of these two in the 10.0 to 12.5 μ region along with a chloroform *versus* chloroform base line are shown in Fig. 1. Solute concentrations were chosen equivalent to the free acid from 9 gr. of each sodium salt extracted into 10 ml. of chloroform; consequently 18 gr. of the 1:1 mixture was used. Each measured absorbance (optical density) is corrected for the absorbance of the "base line" at the selected wave lengths. Each corrected absorbance (A) should be divided by the cell path length (b) in centimeters in order to reduce all observations to absorbance per unit path length (A/b). Factors to correct A/b for interference by the other component and factors for conversion of A/b to milligrams of component per dosage unit are determined as outlined below. (This method has been developed primarily for filled capsules.) These factors will vary considerably from one instrument to another depending on the resolution and response characteristics of the spectrophotometer necessitating individual calibration for each instrument. It is also possible that the factors might vary somewhat with time for a given instrument. The factors can be readily checked by analysis of weighed samples of a standard 1:1 mixture. Although experience has shown these factors to remain valid for periods of several months, analysis of this known mixture requires little time and can be carried out at regular intervals. The factors given below as calculated for the instrument in these laboratories are intended to illustrate their magnitude. Diluents present in the capsules analyzed have been shown experimentally to exhibit no interference in the 10.0 to 12.5 μ region. Products of other manufacturers may contain other diluents so that a quantitative determination of any interference would need to be made on all diluents present in their product.

The absorbance of propylmethylcarbinylallyl barbituric acid at about 10.73 μ obeys Beer-Bouguert's law beyond the concentration range encountered in commercial products. The A/b at 10.73 μ may be corrected for the interference due to isoamylethyl barbituric acid by subtraction of a small constant figure from the A/b value. A conversion factor can be calculated which multiplied by A/b at 10.73 μ gives the amount of propylmethylcarbinylallyl barbituric acid in milligrams per total sample used. Milligrams per capsule can then be obtained by dividing milligrams per sample by number of capsules per sample used. The propylmethylcarbinylallyl barbituric acid factor for this instrument is 26.2.

Isoamylethyl barbituric acid exhibits a rather broad band with the point of strongest absorption at about 11.94 μ. The absorbance of this band is linear with concentration beyond the range encountered. Propylmethylcarbinylallyl barbituric acid also shows considerable absorption at this wave length. It was found inadequate to calculate interference and conversion factors from the spectra of the individual components at 11.94 μ as those factors proved erroneous when both substances were present in the same solution. Rather, the factors for interference and conversion were calculated by weighing mixtures containing the "theoretical" quantity of one component with first 10% more, then 10% less than "theoretical" quantity of the other component. This procedure was then followed starting with the other component at "theoretical" quantity, the resulting data then permitted calculation of the interference factor as well as the factor for conversion to milligrams of isoamyl barbituric acid per capsule. The A/b at 11.94 μ is corrected by subtracting the value obtained by multiplying the propylmethylcarbinylallyl barbituric acid correction factor times the corrected A/b at 10.73 μ as calculated above. With this instrument the correction factor at 11.94 μ for propylmethylcarbinylallyl barbituric acid is 0.617 and the isoamylethyl barbituric acid conversion factor is 38.0. The corrected A/b multiplied by the isoamylethyl barbituric acid conversion factor gives the milligrams per sample. Milligrams per capsule can be obtained by dividing milligrams per sample by number of capsules per sample. It is necessary to determine the correction factors as well as the conversion factors for the instrument to be used.

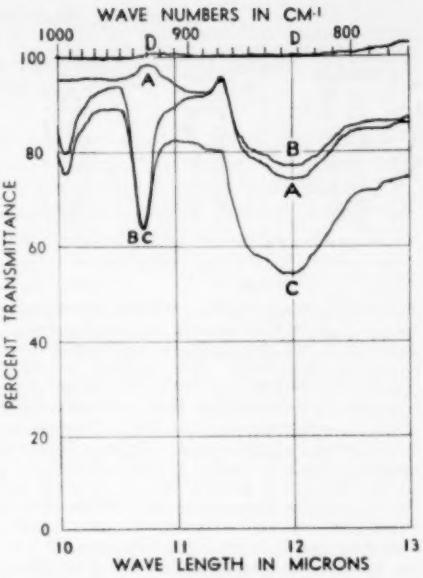

Figure 1.

A. 583 mg. of sodium isoamylethyl barbituric acid carried through assay procedure.
B. 583 mg. of sodium propylmethylcarbinylallyl barbituric acid carried through assay procedure.
C. 583 mg. of each above sodium salt, the combination carried through assay procedure.
D. Chloroform *versus* chloroform base line. Sample cell path length 0.0097 cm.

Procedure for 1:1 Mixtures of Sodium Propylmethylcarbinylallyl Barbiturate and Sodium Isoamylethyl Barbiturate.—Twenty capsules are weighed and the average weight determined. A

TABLE I.—ANALYSES OF WEIGHED MIXTURES

Propylmethylcarbinylallyl Barbituric Acid			Isoamylethyl Barbituric Acid		
Weighed	Found	Recovery, %	Weighed	Found	Recovery, %
22.25	21.80	98.1	22.15	21.88	98.8
22.25	22.27	100.1	22.15	22.59	102.0
22.25	22.16	99.6	22.15	22.59	102.0
44.5	44.9	101.1	44.3	43.5	98.2
44.5	44.9	101.1	44.3	45.2	102.0
44.5	44.7	100.6	44.3	43.1	97.3
89.0	89.1	100.1	88.6	86.2	97.3
89.0	88.4	99.3	88.6	91.8	103.6

number of average weight capsules is taken equivalent to 18 gr. labeled amount total sodium propylmethylcarbinylallyl barbiturate and sodium isoamylethyl barbiturate. The contents of these capsules are carefully transferred to a 25-ml. glass-stoppered Erlenmeyer flask and 10 ml. of distilled water, accurately measured, is added. The contents of the flask are gently swirled to dissolve all soluble material and exactly 1 ml. of 1:1 hydrochloric acid is added. Exactly 10 ml. of chloroform is added and the suspension is vigorously shaken for two to three minutes, or until all particles of precipitated barbituric acids are dissolved. After allowing the mixture to settle for a short time, a portion of the chloroform is transferred by pipette into a 10-ml. glass-stoppered Erlenmeyer flask containing about 1 Gm. of anhydrous sodium sulfate. The contents are shaken to remove suspended water and a portion of the dried solution is then transferred to a rock salt cell of about 0.1-mm. path length. The spectrum for the unknown is recorded from 10.5 to 12.0 μ using chloroform as the comparison solvent. A blank curve of chloroform measured against chloroform is also recorded on the same chart. At each selected wave length, values of per cent transmission for the blank and the sample curves are noted, and absorbance for each is calculated. The blank absorbance is subtracted from that of the sample and the corrected absorbance is divided by the cell path length to give A/b.

Calculation.—

1. $$\frac{\text{Mg./capsule propylmethylcarbinylallyl barbituric acid} = [A/b\ (10.73\ \mu) - \text{constant correction}] \times \text{factor for P.M.C.A. barbituric acid}}{\text{No. capsules used}}$$

2. $$\frac{\text{Mg./capsule isoamylethyl barbituric acid} = [A/b\ (11.94\ \mu) - (\text{Corrected } A/b\ (10.73\ \mu) \times 0.617)] \times \text{factor for I.A.E. barbituric acid}}{\text{No. capsules used}}$$

RESULTS

Table I gives the results of analysis of eight known mixtures. These samples were prepared from weighed amounts of the pure sodium barbiturates. Weight figures in the table show the equivalent of free barbituric acid against which recovery percentages were calculated. Average deviation of the results was 0.8% for propylmethylcarbinylallyl barbituric acid and 2.3% for isoamylethyl barbituric acid. Maximum observed deviation for each component was 1.9% and 3.6%, respectively. This precision of analysis is believed to be adequate for rapid routine control of commercial samples. Table II lists the results of 15 production samples of filled capsules.

TABLE II.—ANALYSES OF PRODUCTION LOTS

Sample	Propylmethylcarbinylallyl Barbituric Acid, Mg.	Theory, %	Isoamylethyl Barbituric Acid	Theory, %
A	22.3	100.2	22.7	102.6
B	43.4	97.6	44.6	100.7
C	45.6	102.5	45.7	103.2
D	43.9	98.7	47.0	106.1
E	45.0	101.2	43.7	98.7
F	44.2	99.4	44.5	100.5
G	46.4	104.2	43.3	97.8
H	45.8	102.9	44.1	99.6
I	90.5	101.8	86.0	97.1
J	91.6	103.0	88.1	99.5
K	83.8	94.2	89.5	101.0
L	84.1	94.6	93.4	105.3
M	85.2	95.8	86.6	97.8
N	86.8	97.6	85.5	96.5
O	89.6	100.8	84.8	95.7

The time required for performance of this method is about forty-five minutes per sample, including the preliminary weighings, the preparation of the sample, and the calculations of results. Where several samples can be prepared and assayed simultaneously, the time per sample is accordingly reduced.

SUMMARY

An infrared method is described for the rapid routine assay of products containing sodium propylmethylcarbinylallyl barbiturate and sodium isoamylethyl barbiturate. Determination of the separate components is made by simple calculation. Tables of results are given and absorption curves are illustrated. Average deviation of results was 0.8 per cent for propylmethylcarbinylallyl barbituric acid and 2.3 per cent for isoamylethyl barbituric acid.

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Report on Antibiotic Activity of Seaweed Extracts*,†

By ROBERTSON PRATT, HENRY MAUTNER, GRACE M. GARDNER, Yi-HSIEN SHA, and JEAN DUFRENOY

Several species of marine algae collected during the fall and spring on the central California coast, yielded extracts that inhibited the growth *in vitro* of one or more of the following species of bacteria: *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (*pyocyanea*). The inhibitory activity of the extracts was not due to iodine. Extracts prepared from plants collected during the winter months were devoid of antibacterial activity.

NEARLY 12,000 species of algae have been cataloged by taxonomists. Approximately 3,000 of these are the larger marine species commonly called seaweeds. A few seaweeds have long been important sources of food, especially in the Orient, and of agar, alginic acid, alginates, iodine, and other products useful in pharmacy and in industry. Formerly, some seaweeds were important as sources of potash in the manufacture of glass and soap.

Species of *Chondrus* (Irish moss) and of *Fucus* (bladderwrack) have had occasional medicinal use. One of the kelps (brown algae) has been used as a source of a heparin substitute (1). But as a group, considering the large number of species available, the algae have been singularly ignored as potential sources of useful medicinal products.

The present paper reports results of a survey of some marine algae of the central California coast as potential sources of antibiotically active substances. For centuries in China several seaweeds have been reputed to possess curative properties in certain infectious processes (2), as well as in some deficiency diseases, but reports of scientific evidence of antimicrobial properties in algae are scarce and meager.

Among the kelps, *Cystophyllum hakodatense* is the source of a pigment that inhibits growth of *Lactobacillus bulgaricus* and of *L. helveticus* (3) and extracts of *Cymathaere triplicata* were found to inhibit growth of *Bacillus subtilis* (4).

Among fresh water species, *Chlorella vulgaris* and *C. pyrenoidosa* biosynthesize a substance that, in appropriate concentrations, limits their own growth and also is capable of inhibiting growth and metabolic activity of other microscopic algae (5–7), as well as of some Gram-positive and Gram-negative pathogenic bacteria (8). *Phormidium uncinatum* and *Scenedesmus quadricauda* also each produce a substance that is capable of inhibiting growth of other microscopic algae (9). No antibacterial properties have been ascribed to the products from *Phormidium* or from *Scenedesmus*. *Spirogyra* species have been reported to markedly reduce the numbers of typhoid, cholera, dysentery, and other colon bacteria in drinking water (10), but the action has been ascribed to liberation of O_2 by the alga and to *p*H changes induced in the water, rather than to biosynthesis of a specific antibiotic substance.

The only practical application that appears to have been made from the observations of antimicrobial activity of fresh-water algae was during World War II when it was observed at several armed services installations in California and Nevada that the effluent from open sewage settling pools heavily inoculated with *Chlorella* sp. was bacteriologically safe for discharge into local streams. In fact, principally during the summer months when high light intensity and the more or less vertical angle of incidence favored growth of the algae, the coliform count was frequently lower in effluent from pools heavily inoculated with *Chlorella* and not chlorinated than it was in effluent from chlorinated pools that contained no *Chlorella* (11).

EXPERIMENTAL

Collection and Preservation.—More than two dozen species of marine algae were collected from rocky portions of the 70-mile stretch of the California coast between San Francisco and Santa Cruz. Samples of sea water were taken at each collection spot and later were tested in the laboratory to make sure that antibacterial activity of seaweed extracts could not be ascribed to contaminants from the ocean water. Collected samples of seaweeds were frozen within thirty minutes after being removed from the ocean and were kept frozen until used in the laboratory.

Extraction.—For routine surveys, both aqueous and immiscible solvent methods of extraction were used. For each species screened, an ether extraction, a cold aqueous extraction, and a hot aqueous extraction were made. A 20% solution of $(NH_4)_2SO_4$ was used for aqueous extractions (see next page).

* Received June 20, 1951, from the University of California College of Pharmacy, San Francisco.
Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.
† The work reported in this paper was financed in part by a generous research grant from Eli Lilly and Co., Indianapolis, Ind.

Organic Solvent Extraction.—Ether emerged from a series of preliminary trials of a large number of solvents as the most generally satisfactory extracting agent. Its principal desirable characteristics are immiscibility with water, sufficiently low boiling point to permit its evaporation by means of reduced pressure at or below room temperature, and lack of toxicity of the dry residue of the solvent for bacteria. The following were eliminated from routine use because they failed in one or more of the above respects: acetone, amyl acetate, dioxane, ethyl alcohol and its mixtures with acetone, methyl alcohol, methylal, polyethylene glycol, methyl isobutyl ketone, tetrahydrofurfuryl alcohol, propylene glycol, chloroform, methyl ethyl ketone, and petroleum ether.

Calcium sulfate, being inert, was considered a satisfactory drying agent for the "wet" extracts. However, relatively large quantities of the drying agent had to be used, and it was soon found that possibly significant amounts of antibacterially active material from the extracts were adsorbed on the $CaSO_4$, as shown by elution of the filtered drying agent and subsequent testing of the eluate. Despite this handicap, $CaSO_4$ was more useful than any other drying agent tested.

The procedure used for ether extraction was as follows:

(*a*) Rinse 100 Gm. of frozen seaweed in tap water to remove adhering sand;

(*b*) Drain seaweed by gravity (without pressure) for ten minutes to remove surplus water;

(*c*) Cut thalli of seaweed into pieces approximately 1 in. long;

(*d*) Extract cut thalli with 500 ml. of peroxide-free diethyl ether (Mallinckrodt fat extraction grade) in Soxhlet extractor for eight to ten hours;

(*e*) Remove material from extractor, separate the aqueous layer, if any, by decantation, and dry the extract over anhydrous $CaSO_4$;

(*f*) Remove drying agent by filtration;

(*g*) Evaporate filtrate to dryness at reduced pressure at or below room temperature;

(*h*) Dissolve or suspend residue in phosphate buffer (*p*H 6.8) for microbiologic testing.

Aqueous Extraction.—The procedure used for aqueous extractions was as follows:

(*a*) Rinse 40 Gm. of frozen seaweed in tap water to remove sand;

(*b*) Cut thalli into small pieces and place in Waring blendor with 300 ml. of a 20% solution of $(NH_4)_2SO_4$ for ten minutes;

(*c*) After blending, filter immediately for cold extraction, or heat to 80° on a water bath and maintain at 80° for fifteen minutes and then filter for hot extraction

(*d*) Adjust *p*H of filtrate, if necessary, to 6.8 for microbiologic testing. The filtrates were tested directly and also after concentration.

Since the aqueous extracts invariably were very viscous, filtration was difficult. However, use of gray crepe "Sugar Filtration" filter paper at atmospheric pressure was quite satisfactory and was superior to vacuum filtration which tended to clog the pores of this and of all other types of paper tried.

Microbiologic Testing.—The several extracts and samples were tested for antibacterial activity by the cylinder plate technique using *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (*pyocyanea*) as routine test organisms.

It was decided empirically not to investigate further any species of seaweed which under the standard extraction procedures failed to yield an extract, in at least one of the methods, that would produce a clear zone 12.0 mm. or more in diameter against at least one of the test organisms.

Occasionally, additional tests were made, using *Bacillus subtilis* and *Proteus vulgaris* as test organisms. A few extracts were tested against *Trichophyton mentagrophytes*. All tests against this fungus were negative.

Most tests were made with extracts, but routinely in starting to work with any species of seaweed, the seaweed itself was tested by placing disks of tissue cut from it or a sample of the crushed tissue on the seeded agar plates. These tests were uniformly negative.

In all tests with extracts, controls consisted of the organic solvents or of a 20% solution of $(NH_4)_2SO_4$ handled in exactly the same way as the extracts, but containing no extract.

RESULTS

Winter collections of the seaweeds studied were uniformly devoid of activity against the bacteria used as test organisms, but autumn and spring collections of several species yielded extracts with definite antibacterial activity. This points to seasonal variations in chemical composition (see below). The species which yielded antibacterially active extracts, when collected at the proper season, are shown in Table I, along with the average diameters of the inhibition zones their different extracts produced against three species of bacteria.

Chlorophyta.—*Ulva* (*Enteromorpha*) *Linza* was the only green alga of several tested that yielded extracts with demonstrable antibacterial activity. Cold aqueous extracts of specimens collected in February and March showed moderate activity against *S. aureus*. The active material apparently is unstable, since the "cold" extracts, which when fresh gave appreciable inhibition zones on seeded assay plates, were totally inactive after storage at 2–4° for several days. "Hot" aqueous extracts were inactive. Ether extracts showed moderate activity against *Ps. aeruginosa*.

U. Linza is abundant on rocks between the 2.0 foot and low tide levels of the Pacific coast from Alaska to Lower California.

Another green alga (*Cladophora trichotoma*) and a marine spermatophyte (*Phyllospadix* sp.) also were extracted, but neither showed activity against the test organisms.

Phaeophyta.—*Egregia Menziesii* is widely distributed along the Pacific coast, extending from Alaska to Southern California. The species is somewhat indistinct and shows marked morphologic variations in different sectors of its range and even in a given sector at different seasons of the year. In fact, so variable were the morphologic characteristics of specimens collected at Moss Beach at different seasons of the year (Fig. 1) that an expert algologist[1]

[1] We are indebted to Dr. George F. Papenfuss, Curator of Algal Collections, University of California Herbarium, for his identification of several of the species of marine algae discussed in this paper.

TABLE 1.—DIAMETERS OF INHIBITION ZONES PRODUCED BY EXTRACTS OF SEAWEEDS ON AGAR PLATES SEEDED WITH DIFFERENT TEST ORGANISMS

Class and Species of Seaweed	Extracts and Organisms: Ether — *S. aureus*	Ether — *E. coli*	Ether — *Ps. aeruginosa*	Cold Aqueous — *S. aureus*	Cold Aqueous — *E. coli*	Cold Aqueous — *Ps. aeruginosa*	Hot Aqueous — *S. aureus*	Hot Aqueous — *E. coli*	Hot Aqueous — *Ps. aeruginosa*
Chlorophyta									
Ulva Linza	0.0	0.0	13.8	13.6	10.9	10.3	0.0	0.0	0.0
Phaeophyta									
Egregia Menziesii	13.4 / 15.3 [b]	9.5	10.0 / 23.0	9.6	9.3	12.3	12.5	15.0	10.0
Macrocystis pyrifera	9.0 / 12.0 [b]	11.5	0.0	0.0	0.0	0.0	0.0	12.0[d]	10.3[d]
Nereocystis leutkeana	10.5 / 13.3 [b]	11.0[d]	8.5	..	..	..	0.0	10.8[d]	0.0
Pelvetia fastigiata	8.7	10.2	0.0	8.5	0.0	10.8[d]	8.7	8.7	11.5[d]
Postelsia palmaeformis[c]	13.5	12.0[d]	0.0	9.0	8.5	0.0	10.0	10.5[d]	16.5[d]
Rhodophyta									
Callophyllis megalocarpa	10.0	0.0	9.7[d]	11.2	10.2	12.0	10.0	11.3– / 13.0– [b]	0.0
Halosaccion glandiforme	10.7	0.0	10.5	10.5	0.0	10.1	10.0	9.5	12.0
Iridophycus flaccidum	10.3	10.8	12.8	10.1	11.0	9.0	10.5	10.5	12.5

[a] Generally, controls prepared by treating ether or 20% aqueous solutions of $(NH_4)_2SO_4$ in exactly the same manner as the extracts, except that no seaweed was added, gave negative tests, i. e., they did not inhibit growth of the test organisms. Occasionally, however, the controls indicated slight inhibition due to solvent residues. The maximum inhibition observed in any of the controls was as follows. All figures in this table are in mm.

	S. aureus	*E. coli*	*Ps. aeruginosa*
Ether	0.0	9.0 static	11.5 static
$(NH_4)_2SO_4$	8.5 clear	10.5 clear	10.0 clear

[b] Occasionally the inhibition area consists of two distinct regions, one clear (complete inhibition) and outside of this a secondary area of partial inhibition. In such instances two figures are given; the smaller one represents the diameter of the clear zone, and the larger one the diameter of the combined inhibition zones (clear zone + static zone).

[c] Dioxane extractive of this species also possessed pronounced antibacterial activity (see Fig. 3). The photograph shows that the dioxane control also had some activity, although much less than the extract.

[d] Static zone only.

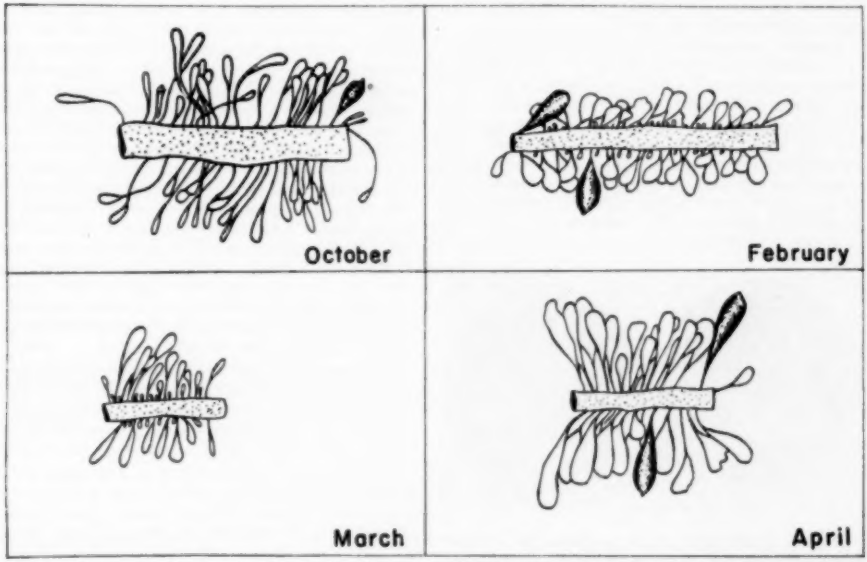

Fig. 1.—Segments of *Egregia Menziesii* collected at different times at Moss Beach, Calif. Note the changes in size and shape of the fronds at different seasons. (Approximately one-half actual size.)

Fig. 2.—Collection of *Postelsia palmaeformis* at low tide, showing the palmlike appearance of the plants which consist of distinct frond and stipe portions.

was consulted to establish the taxonomic identity of the several samples.

Hot aqueous extracts and ether extracts of material collected in October and in the following April showed distinct activity against *S. aureus*, but extracts of specimens collected in February were totally inert antibacterially (see below).

Postelsia palmaeformis is one of the most distinctive seaweeds of the Pacific coast. Its palmlike appearance is unique among the Pacific algae (Fig. 2). Stipes and fronds of *P. palmaeformis* were tested separately. No antibiotic activity was detected in either portion of unextracted plants or in extracts prepared from the stipes. However, ether extractives and dioxane extractives of fronds from plants collected in October markedly inhibited the growth of *S. aureus* and *Ps. aeruginosa*, respectively (Fig. 3). Other test organisms were affected to a lesser extent. Soon after the October collections were made, severe storms of hurricane proportions destroyed remaining plants and no specimens could be collected during the following winter and spring months so that it was impossible to follow seasonal variations in extracts from this species.

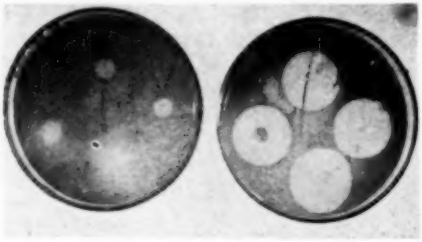

Fig. 3.—Inhibition zones produced by dioxane extract of fronds of *Postelsia palmaeformis* on assay plate seeded with *Pseudomonas aeruginosa*. Control is on left; extract is on right.

Rhodophyta.—Three species of red algae yielded extracts that possessed some activity against one or more of the test organisms.

Callophyllis megalocarpa grows on sheltered or exposed rocks between the mean low tide and 1.5-foot levels. Cold aqueous extracts of this species showed weak activity against *S. aureus;* neither the hot aqueous nor the ether extractive was active.

Halosaccion glandiforme is a red alga that grows attached to rocks in the intertidal zone. It consists of sacs, ranging in length from a few millimeters to 8–10 cm., that contain varying volumes of liquid. The fluid removed from *Halosaccion* by carefully snipping off the tip and squeezing the sac, when tested directly on seeded test plates, inhibits somewhat the growth of *S. aureus*, *E. coli*, and *Ps. aeruginosa*. Methylal and acetone extractions of sac walls of *Halosaccion glandiforme* after removal of the sac contents gave some inhibition of *S. aureus* and of *Ps. aeruginosa*, although ether extracts uniformly were negative or gave only minimal inhibition.

Iridophycus flaccidum is common along the California coast from Crescent City to Point Sur. It grows principally in the low littoral, several blades usually being attached to the rocks by a common holdfast. The fronds are quite flaccid and, when under water in strong light, are highly iridescent, from which facts the species derives its name.

Ether extractives of *I. flaccidum* collected in October markedly inhibited growth of *Ps. aeruginosa*. Aqueous extracts were less active.

Seasonal Variation in Antibiotic Activity.—The work reported in this paper was begun in October, 1950. Subsequent collections made during the winter yielded extracts that were negative antibacterially, as was mentioned above. Fortunately, enough *Postelsia*, *Egregia*, and *Iridophycus* from October collections still remained in the deep freeze to permit retesting. In each instance, the new extracts showed activity approximately equal to that of the original preparations. Extracts from March, 1951, collections showed slight antibacterial activity and extracts from April collections were beginning to approach the activity of extracts from the previous October collections. These observations lead to two conclusions, namely, that there are pronounced seasonal variations in the chemical composition of these seaweeds and that their antibiotically active constituents are stable in the frozen state *in situ* for at least five months.

This result was not unexpected, since numerous other investigators have demonstrated pronounced seasonal changes in the yields of algin, laminarin, protein, ash, etc., from several species of marine algae, a fact which we have confirmed (see below).

In passing, it is noteworthy that at least three hundred and fifty years ago the Chinese apparently were aware of the fact that the chemical composition of seaweeds depends on the time of year. In a Chinese pharmacopoeia of 1598, directions for preparing extracts of Hai-Tsao (*Sargassum siliquastrum*, one of the brown algae) for medicinal use require that

plants collected in summer be soaked for two days and those collected in winter be soaked for three days.

In the present study, analyses for alginic acid, laminarin, proteins, ash, and other constituents were made monthly, concomitantly with the antibiotic studies. The detailed results of the analyses which in general confirm the observations of other workers (12) will be reported later. It will suffice for the present to state that there appears to be a relationship between antibiotic activity and alginic acid and protein content. This is merely a statement of empirically observed fact and is not meant to imply that there is a significant biochemical or metabolic relationship between these constituents and the biosynthesis of antibiotically active compounds or complexes.

DISCUSSION

It is apparent that, except during the winter months, several species of seaweeds contain an extractable substance (or substances) that inhibits the growth *in vitro* of one or more of the test organisms that were used.

A number of the marine algae, especially the browns, have long had a reputation as sources of therapeutically useful amounts of iodine. However, no evidence could be found to indicate that the antibacterial activity of the extracts was due to the presence of iodine.

Fucus vesiculosus is notable among the seaweeds for its iodine content, but extracts of this species were negative antibacterially.

It is noteworthy also that extracts of fronds always were more active than those from stipes (which usually were negative) although it has been reported that the iodine content is generally higher in the stipes than in the fronds of marine algae (13). Moreover, iodine generally does not occur in seaweeds in the form of free iodine, but rather as inorganic salts or in other forms unsuitable for antibacterial action.

Furthermore, the extraction methods used, especially with organic solvents, would not be expected to yield dry residues containing iodine, and neither the extracts nor the crushed seaweeds gave positive tests with starch-iodide paper, which is an exceedingly sensitive test for the presence of free iodine.

The control tests with sea water and with sea water extracts showed that the antibacterial activity of the extracts cannot be ascribed to the presence of ocean-borne bacteriophage or other contaminants adhering to the tissues of the algae.

Therefore, it seems apparent that several species of seaweeds found along the California coast biosynthesize an extractable substance (or substances) that inhibits (or inhibit) the growth *in vitro* of one or more of the test organisms that were used. Work is now in progress to attempt identification of the antibacterial material extracted from the different seaweeds and to accumulate enough of it for more extensive tests *in vitro* and *in vivo*.

SUMMARY

Several species of marine algae that grow along the central California coast yield ether extracts or aqueous extracts that inhibit the growth *in vitro* of one or more of the following bacteria: *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. There was no evidence that the antibacterial activity of the extracts could be ascribed to the presence of iodine.

Marked seasonal variation occurred in the chemical composition of the seaweeds and in antibacterial activity of their extracts. During the winter, alginic acid and protein content tended to be higher and antibacterial activity to be lower than at other seasons, but this is not meant to imply a causal relationship between the two.

The following is a list of the seaweeds that yielded antibacterially active extracts:

Chlorophyta, or green algae
Ulva Linza
Phaeophyta, or brown algae (kelps)
Egregia Menziesii
Macrocystis pyrifera
Postelsia palmaeformis
Rhodophyta, or red algae
Callophyllis megalocarpa
Halosaccion glandiforme
Iridophycus flaccidum.

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The Rearrangement of Codeine to Dihydrocodeinone*

By MANUEL M. BAIZER, KURT S. ELLNER, and ATHANASIOS LOTER†

A study was made of the factors affecting the catalyzed rearrangement of codeine to dihydrocodeinone in acidic aqueous medium. A maximum yield of about 50 per cent of pure dihydrocodeinone was obtained. Certain provisional conclusions concerning the course of the reaction are discussed.

IT HAS been claimed in several German patents (1) that codeine can be isomerized to dihydrocodeinone in 70–95 per cent yields by means of a noble metal catalyst in an acidic aqueous or in an alcoholic medium.

There is a certain vagueness in the experimental descriptive sections of the German patents concerning reagent concentrations. It is also not quite clear whether the weight of crude or of recrystallized product was used as a basis for yield calculation. We have therefore examined the conditions of this rearrangement rather closely. In no case did we achieve yields higher than ca. 50 per cent in an individual experiment.[1,2]

When several attempts to rearrange codeine in alcoholic medium failed to give evidence of any reaction whatsoever, we abandoned this approach completely;[3] all rearrangements reported here were performed in acid medium.

Early in this investigation it became evident that a reliable method of determining the quantity of pure dihydrocodeinone formed in each run would have to be developed before variations in reaction conditions could be evaluated. Recrystallization of the crude product from alcohol (1) did not give dihydrocodeinone meeting the standards of New and Nonofficial Remedies (6), and in addition left varying amounts in mother liquors. Likewise recrystallization of crude dihydrocodeinone-sodium bisulfite and subsequent liberation of the purified alkaloid (7) was not satisfactory. The procedure finally adopted is described in the Experimental section; it was reasonably rapid and completely reproducible. All yields given in this report are based on results obtained by that evaluation procedure.

EXPERIMENTAL AND RESULTS

Table I is a summary of our most significant exploratory experiments. Individual runs or groups of runs will be cited in illustration of the conclusions we reached concerning the effect upon the yield of product of systematic changes in the reaction conditions (Procedure 1).

Procedure 1

In all cases 30.0 Gm. of codeine (anhydrous basis), 12.5 Gm. of catalyst A,[4] and 200 cc. of acid solution comprised the reaction mixture. After completion of the reaction under the conditions cited in Table I, the mixture was filtered, the catalyst washed with dilute acid, and the combined acid solutions made alkaline with sodium hydroxide to *p*H 10 and extracted with a total of 875 cc. of benzene. The benzene extracts were washed with water and then stirred with 50 cc. of 38% aqueous sodium bisulfite for one hour The precipitated dihydrocodeinone-sodium bisulfite was removed by filtration, air-dried, and evaluated by the procedure described below.

Acid Strength.—Runs 1–4 demonstrate that a strong acid is necessary for successful rearrangements.

Acid Concentration.—Run 5 leads to the conclusion that, provided a strong acid is used, the concentration of acid may be varied considerably without great effect upon the yield.

Temperature.—Run 4 *vs.* run 6 illustrates that the rearrangement is best conducted at reflux temperature.

Atmosphere.—In runs 7 and 8, nitrogen and hydrogen, respectively, were bubbled through the reaction mixture; the effect in both cases was deleterious.

Stirring.—Run 9, in which stirring was used, gave poor results. All other runs were done without stirring.

Time.—Run 10 illustrates that a considerable proportion of the rearrangement has occurred within the first twenty minutes of reflux. It is definitely advantageous, however, to continue the reaction beyond one hour.

On the basis of the above findings, the reaction conditions were standardized (Procedure 2) and the study restricted to an evaluation of the effects of changes of catalyst. As seen in Table II, these changes most profoundly influenced the yield.

* Received May 23, 1951, from the Research Laboratory of the New York Quinine and Chemical Works, Inc., Brooklyn, N. Y.

† The suggestions and criticisms of Dr. W. G. Bywater, S. B. Penick and Co., are gratefully acknowledged.

[1] Similar results were evidently obtained by Rapoport, *et al.* (2). In a private communication Dr. Rapoport has informed us that he does not intend to publish the results of his study of this rearrangement.

[2] Since the submission of this manuscript, there has appeared an abstract of a Japanese paper [*J. Pharm. Soc. Japan*, **62**, 352(1942); *Chem. Abstr.*, **45**, 5167(1951)] which claims that codeine is rearranged to dihydrocodeinone in 75–79% yields using 10% palladium black as catalyst, 6% hydrochloric acid as medium, a temperature of 50–80°, and a current of hydrogen.

[3] The one exception was the unexpected finding that when codeine is heated with Raney nickel in toluene containing cyclohexanone (3), it is rearranged to dihydrocodeinone in 42% yield. Like Findlay and Small (3) we had investigated this reaction in an attempt to prepare codeinone which could then have been hydrogenated to dihydrocodeinone (4, 5).

[4] This catalyst was 5% palladium on activated charcoal, lot No. 860, Baker & Co., Newark, N. J.

TABLE I.—REARRANGEMENT OF CODEINE TO DIHYDROCODEINONE: CATALYST CONSTANT

Run No.	Acid: Species	Acid: Vol. (Cc.)	T., °C.	Time, Hr.	Alkali-Sol. Products, %[a]	Yield of Dihydrocodeinone, %
1	HOAc	10.8	Reflux	1.25	18.0	0.91
2	H_3PO_4	4.0	Reflux	1.25	17.3	0.52
3	HCl	15.3	Reflux	1.25	26.0	44.4
4	H_2SO_4	5.0	Reflux	1.25	17.5	47.0
5	H_2SO_4	10.0	Reflux	1.25	17.5	43.2
6	H_2SO_4	5.0	70	1.25	13.6	35.1
7[c]	H_2SO_4	5.0	90–93	3	14.6	33.8[b]
8[d]	H_2SO_4	5.0	Reflux	1.25	14.9	38.0
9	H_2SO_4	5.0	Reflux	1.25	15.0	36.1
10	H_2SO_4	5.0	Reflux	0.33	15.7	32.1

[a] Weiss, U., and Weiner, N., *J. Org. Chem.*, **14**, 194(1949) report the isolation of thebainone.
[b] Slow stream of hydrogen bubbled through reaction mixture throughout rearrangement.
[c] Product had to be twice purified over alumina.
[d] Slow stream of nitrogen bubbled through reaction mixture throughout rearrangement.

Procedure 2

The reaction mixture, consisting of 30.0 Gm. of codeine (anhydrous basis), 5.0 cc. of concentrated sulfuric acid, 195 cc. of water, and the quantity and type of catalyst indicated in Table II was heated under reflux without stirring for one and one-quarter hours. The isolation of product, etc., followed Procedure 1.

Quantity of Catalyst.—Runs 11 and 12 show that either increasing or decreasing the quantity of catalyst has a deleterious effect upon the yield obtained in run 4. The increase in the quantity of catalyst was associated with a significant increase in the amount of alkali-soluble products formed.

Concentration of Metal on Carrier.—Runs 13 and 14 demonstrate that an increase in the concentration of noble metal on the same carrier results in a decrease in yield. There was, surprisingly, no increase in the extent of formation of alkali-soluble products. See reference in footnote *a* to Table I.

Nature of Noble Metal.—Platinum, at least in our hands, gave poorer results (run 15) than palladium, and the addition of a small amount of platinum to a good palladium catalyst (run 16) likewise was harmful. Ruthenium, on the other hand, gave excellent results (run 17).

Activity Number.—We could find no correlation between the efficiency of a catalyst as measured by its performance in a hydrogenation and its ability to catalyze the rearrangement of codeine.

Bulk of Catalyst.—The results of several runs favored the conclusion that a bulky catalyst was desirable. Run 21 was made with a dense (i. e., *not* bulky) catalyst.

Evaluation of the Dihydrocodeinone-Sodium Bisulfite

A representative sample (15.0 Gm.) was suspended in 15 cc. of water in a 250-cc. beaker. Ninety cubic centimeters of ethylene chloride was added, stirring was started, and 15 cc. of concentrated ammonia was run in slowly. After ten minutes of stirring, the mixture was transferred to a separatory funnel and the layers separated. The aqueous solution was further extracted with two 45-cc. portions of ethylene dichloride. The combined organic extract was washed with water and dried over anhydrous sodium sulfate.

A column of activated alumina[5] 0.75 in. in diameter and 6 in. high was wetted with dry ethylene dichloride. Then the ethylene dichloride solution of the alkaloids was passed through by gravity, followed by 200 cc. of fresh dry solvent. The combined effluents were made to 400 cc. and a 25-cc. aliquot was evaporated to dryness; the residue was dried at 110° for two hours and weighed.

Let x = weight in Gm. of the dihydrocodeinone in 25 cc.; y = total weight in Gm. of the dihydro-

[5] Alumina F-20, Aluminum Corporation of America, was used.

TABLE II.—EFFECT OF CATALYST VARIATIONS UPON YIELD

Run No.	Catalyst: Metal, %	Catalyst: Carrier	Catalyst: Gm.	Catalyst: Lot No.[a]	Activity No.[b]	Yield of Dihydrocodeinone, %
4	Pd (5)	C	12.5	860	915	47.0
11	Pd (5)	C	6.25	860	915	32.3
12	Pd (5)	C	25.00	860	915	34.0
13	Pd (10)	C	12.5	[c]	..	24.1
14	Pd (15)	C	4.17	423	355	35.1
15	Pt (5)	C	12.5	514	785	<10.5
16	Pd (5)	C	12.0	860	915	35.3
	Pt (5)	C	0.5	514	785	45.3
17	Ru (5)	C	12.5	363A	..	
18	Pd (5)	C	12.5	845	965	36.3
19	Pd (5)	C	12.5	388	820	35.6
20	Pd (5)	C	12.5	380	465	33.3
21	Pd (5)	C	12.5	425	..	24.9

[a] Baker and Co., Newark, N. J.
[b] Supplied by the manufacturer and denotes the cc. of hydrogen absorbed in five minutes in the hydrogenation of double-distilled nitrobenzene in glacial acetic acid at room temperature using 500 mg. of catalyst. *Cf.* Hoffmann, *et al.* (8).
[c] Prepared in this Laboratory by the method of Organic Syntheses (9).

codeinone-sodium bisulfite. Then 3.56 *xy* is the % yield of dihydrocodeinone in the run.

Attempts to Dehydrogenate Dihydrocodeine to Dihydrocodeinone

Using 5% Palladium-on-Charcoal.—In a 100-cc. three-necked flask equipped with mercury-sealed stirrer, thermometer, and reflux condenser was placed 5.65 Gm. of dihydrocodeine dihydrate, an equal weight of 5% palladium-on-charcoal, and 20 cc. of xylene. The mixture was heated first to drive off water of hydration and then under reflux for four and one-half hours. It was cooled and filtered. The filtrate was shaken for thirty minutes with 17 cc. of a saturated aqueous solution of sodium bisulfite. No precipitate formed. The aqueous layer was separated and made alkaline. The precipitated gum was removed, dissolved in acid, and reprecipitated, yielding 5.34 Gm. of dihydrocodeine dihydrate.

Using Copper Bronze.—Dihydrocodeine dihydrate (5.60 Gm.) was dissolved in 100 cc. of diisopropylbenzene and heated under reflux for three hours with 1.0 Gm. of copper bronze. The mixture was cooled, filtered, and diluted with benzene to a volume of 135 cc. The filtrate was shaken for one hour with 8 cc. of 38% sodium bisulfite. After chilling, the lower layer was separated, made alkaline, and thoroughly extracted with chloroform. The chloroform solution was evaporated to dryness; the residue, 0.92 Gm., did not form an oxine and was therefore not dihydrocodeinone.

From the organic layer 4.50 Gm. of nonketonic alkaloid was recovered in the usual manner.

DISCUSSION

The catalyzed isomerization of codeine to dihydrocodeinone appears to be a complex process involving more than one mechanism. The mixture at the end of a run contains a multiplicity of products. The following conclusions and hypotheses are consistent with the facts:

1. The reduction of codeine to dihydrocodeine by the hydrogen absorbed on the catalyst is *not* the first step of the reaction. Dihydrocodeine is not dehydrogenated to dihydrocodeinone under "codeine rearrangement conditions" nor indeed under more vigorous conditions (palladium-on-charcoal in hot xylene; copper bronze at elevated temperatures).

2. The dehydrogenation of codeine to codeinone is a plausible first step, followed by:

(*a*) Reduction of codeinone to dihydrocodeinone (5).

(*b*) Transformation of a portion of the codeinone in the acidic medium to phenolic products (3).

(*c*) Hydrogenolysis of the ether bridges of codeinone and dihydrocodeinone, respectively, by means of H_2/Pd. We have found, for example, that dihydrocodeinone under "codeine rearrangement conditions" is converted to the extent of about 15 per cent into alkali-soluble products.

3. The formation of dihydrocodeine as a by-product,[6] probably by reduction of a portion of the original codeine by means of H_2/Pd, precludes the possibility that intramolecular transfer of hydrogen from the—CHOH group of codeine to the double bond or the oxygen bridge is the sole operating mechanism.

4. The efficacy of the catalyst as a dehydrogenating rather than a hydrogenating aid seems critical. Unfortunately the isolated dehydrogenation of codeine to codeinone has not yet been achieved. (*Cf.* footnote 3 and literature citations therein.)

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[6] Isolated in this Laboratory previously from the reaction mixture.

REMINGTON MEDAL AWARD

The Remington Medal, Pharmacy's highest award, will be presented to Dr. Hugo H. Schaefer at a dinner to be held in his honor on Tuesday evening, December 11, in the Grand Ballroom, Waldorf-Astoria Hotel, New York City.

The Remington Medal is presented by the New York Branch of the AMERICAN PHARMACEUTICAL ASSOCIATION to the individual who, during the past year or over a period of years, has made outstanding contributions to the advancement of Pharmacy.

Dr. James H. Kidder, Dean of Fordham University College of Pharmacy, will serve as toastmaster. Tickets for the dinner ($10 per person) may be obtained by writing to the dinner chairman, Professor Frank J. Pokorny, 115 West 68th Street, New York 23, N. Y.

A Comparison of the Chronic Toxicities of Synthetic Sweetening Agents*

By O. GARTH FITZHUGH, ARTHUR A. NELSON, and JOHN P. FRAWLEY

Data are given on the chronic oral toxicities of the synthetic sweetening agents, saccharin, sodium cyclohexyl sulfamate, dulcin, and P-4000. The results indicate that these four substances may be divided into a toxic group and a relatively nontoxic group. Dulcin and P-4000 were toxic to rats at dosage levels of 0.1 per cent and above, whereas saccharin and sodium cyclohexyl sulfamate had only slight effects at a dosage level of 5 per cent.

THE DISCOVERY and proposed use of two new synthetic noncaloric sweetening agents, sodium cyclohexyl sulfamate (hereinafter called cyclamate sodium) in this country (1), and P-4000 (1-*n*-propoxy-2-amino-4-nitrobenzene) in the Netherlands (2), led us to compare the relative chronic toxicities of these substances with the older sugar substitutes. The following experiments were designed to evaluate the relative safety of cyclamate sodium, P-4000, saccharin, and dulcin (4-ethoxy-phenylurea).

METHOD

Four parallel experiments were conducted in which groups of weanling albino rats (twenty-one days), from our colony of Osborne-Mendel strain and equally divided between the sexes, were started on diets containing one of the above-named sweetening agents. A total of 528 animals was used.

In the individual experiments, each of which included the dosage levels of a single substance and a control group, the number of rats per group was 20 except for the experiment with P-4000, which had 24. All animals were randomized into groups according to litter mates. The rats were started on dosage levels of 1, 0.5, 0.1, and 0.01% for each substance. Additional dosage levels of 5% saccharin and cyclamate sodium, 0.25% dulcin, and 0.25 and 0.001% P-4000 were used. Ground commercial rat biscuits with 1% added cod-liver oil served as a basic diet. The sweetening agents were mixed with the basic diet by means of a rotary batch mixer. All animals were kept in individual cages in a room with the temperature and humidity controlled for the duration of the experiment and were given free access to their respective diets and water. The weights of individual animals and their food consumption were determined at weekly intervals. Most animals were allowed to live out their natural life span; however, those living at the end of two years on the experiment were sacrificed.

RESULTS

Since the four experiments were started at the same time and were part of the same study, they will be discussed together.

* Received June 28, 1951, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

The Effect on Growth Rate and Food Consumption—An inspection of Table I shows that dosage levels of 0.5 and 1% dulcin and P-4000 retarded the growth rate significantly. Likewise the animals on 5% saccharin and cyclamate sodium were smaller than the controls. These differences were not significant ($p < 0.05$) for the groups of males on cyclamate sodium and females on saccharin. All other dosage levels of each substance showed no retardation of growth. Individual growth curves from the same litter and the composite growth curves of the several groups showed little variation during the fast-growing period and during the plateau period. Analyses, therefore, for the gain in weight for the first twelve weeks and for the first twenty-six weeks of the experiment showed the same statistical differences as those for fifty-two weeks (Table I).

When the data for the weekly food consumption of each group during the first twenty-six weeks and during the second twenty-six weeks of the experiment were analyzed, the differences between the control and experimental animals were not statistically significant. A slight increase in food intake occurred with groups of animals on 5% cyclamate sodium and saccharin, and this can be accounted for by the amount of bulk noncaloric material added to the diet.

The Effect on Mortality.—Statistical tests cannot be applied to the mean survival time since all surviving animals were sacrificed at the conclusion of two years on the experiment. Most of the deaths during the first year, except for a group on dulcin, were distributed throughout all groups and in most cases represented animals killed because of middle ear infection. The single exception was the group of animals on 1% dulcin, which produced a 25% mortality during the first year. When the number of animals surviving at eighteen months and at two years were selected as estimates of relative toxicity, the dosage levels of 0.5% dulcin and 1% P-4000 also increased the mortality rate. Other dosage levels had no significant effect on mortality. The mortality rates for control animals at one year and two years were 14% and 68%, respectively.

The Effect on Organ Weights.—As is shown in Table II, dulcin and P-4000 increased the relative size of livers, kidneys, and spleens of the animals on the higher dosage levels. This was most evident in the case of the spleens in animals receiving 1% dulcin, where the spleens weighed up to 4.6 Gm. compared with an average control weight of 0.9 Gm. Spleen, liver, and kidney weights were significantly

TABLE I.—MEAN GAIN IN WEIGHT OF RATS FED DIETS CONTAINING SYNTHETIC SWEETENING AGENTS FOR ONE YEAR

Substance	Dosage, %	No. of Animals	Sex	Mean Gain in Wt., Gm.
Saccharin	Control	7	M	475.4 ± 27.25
		9	F	245.4 ± 8.93
	1.0	10	M	467.0 ± 22.24
		10	F	270.1 ± 12.93
	5.0	9	M	409.5 ± 11.23[a]
		9	F	226.6 ± 10.16
Cyclamate sodium	Control	9	M	439.7 ± 24.25
		11	F	286.4 ± 11.14
	1.0	6	M	468.8 ± 24.96
		9	F	241.7 ± 12.22
	5.0	9	M	408.5 ± 8.08
		7	F	209.3 ± 6.94[b]
Dulcin	Control	7	M	492.1 ± 29.80
		7	F	289.2 ± 15.01
	0.25	8	M	462.8 ± 22.86
		7	F	251.8 ± 24.87
	0.1	9	M	499.7 ± 24.63
		9	F	277.2 ± 6.80
	0.5	10	M	418.0 ± 15.09[a]
		7	F	204.4 ± 7.90[a]
	1.0	7	M	337.0 ± 18.73[b]
		7	F	174.0 ± 8.15[b]
P-4000	Control	10	M	478.9 ± 19.53
		9	F	256.7 ± 15.77
	0.25	10	M	489.8 ± 23.09
		10	F	246.8 ± 7.73
	0.1	10	M	514.7 ± 23.80
		9	F	254.2 ± 13.46
	0.5	11	M	403.5 ± 14.13[a]
		12	F	235.3 ± 9.61[a]
	1.0	9	M	397.3 ± 18.58[b]
		12	F	208.1 ± 5.72[b]

[a] $p < 0.05$.; [b] $p < 0.01$.

different from the controls at 0.5% dulcin, but not at 0.25% or less. At dosage levels of 1.0 and 0.5%, P-4000 significantly increased the relative weights of liver and kidney, while increase in splenic weight was not significant. Certain correlations and explanations of the above-mentioned weight increases will be found with the description of the microscopic pathology. Saccharin and cyclamate sodium had no effect on organ weights.

Pathology.—Of the 528 treated and control animals started on the experiment, all but a few had a gross examination of their tissues at autopsy, and 407 were examined microscopically. Detailed examination was done in 185 instances and routinely included hematoxylin-eosin stained paraffin sections of lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, kidney, adrenal, thyroid, testis (or uterus and ovary), leg muscles, leg bones, and bone marrow; the remaining 222 were usually limited to liver, kidney, and (in males) testis. Various special stains were done in a limited number of instances.

Dulcin.—At the higher dosage levels, two effects of dulcin were frequent, and distinctly noticeable grossly. Liver tumors began appearing in rats autopsied after fifty-nine weeks, and eventually involved 10 of the 1% rats, 2 at 0.5%, and 1 at 0.1%. These tumors were up to 2.5 cm. in diameter, although usually about 8 mm. in diameter, and were sometimes multiple. They accounted for most, if not all, of the increased average organ weight. Enlargement and a purplish darkening of the spleen were noted as early as thirteen weeks on the experiment. The incidence and degree of this lesion, present at levels as low as 0.1%, diminished more gradually with decreasing dosage than did the liver tumors.

On microscopic examination the liver tumors were seen to be hepatic cell adenomas, composed of irregular cords and masses of cells slightly to moderately larger than the surrounding hepatic cells, and markedly but by no means completely similar to them in shape and staining quality. Some of the larger tumors were histologically malignant. Apart from the tumors, the livers showed no other direct effect of the dulcin. There were certain indirect effects, namely hepatic cell atrophy, Kupffer cell hemosiderosis and bile duct proliferation, all in relatively slight degree. The hemosiderosis was the most significant of these; it was very slight at 0.25%, slight at 0.5%, and slight to moderate at 1.0%.

The enlargement and darkening noted grossly in the spleen were associated microscopically with chronic congestion and to a lesser extent with hyperplasia of the pulp and an increased amount of hemosiderin. At 0.01% these changes were absent; at 0.1% they were present in a slight degree at seventy-nine weeks and thereafter, whereas at 0.25% they were definite in the first animal autopsied at twenty-seven weeks, and as time and dosage increased there was a parallel increase in the microscopic changes related to the enlargment and darkening. At the two highest dosage levels these generalized changes were sometimes overshadowed by localized changes. The local lesions appeared in the animals on 0.25% dulcin at fifty-eight weeks and thereafter, and consisted of rounded areas about

TABLE II.—LIVER, KIDNEY, AND SPLEEN WEIGHTS OF RATS FED THE HIGHER DOSAGE LEVELS OF SWEETENING AGENTS FOR ONE YEAR OR MORE

Substance		Mean Weight in Gm./Kg. of Body Weight		
		Liver	Kidney	Spleen
Control		30.4 ± 1.5	7.2 ± 0.4	2.0 ± 0.3
Saccharin	5%	29.4 + 1.9	8.5 ± 0.4	2.1 ± 0.2
	1%	38.4 ± 1.9	8.5 ± 0.5	2.1 ± 0.2
Cyclamate	5%	27.8 ± 1.9	7.6 ± 0.5	2.1 ± 0.2
sodium	1%	26.7 ± 1.9	7.5 ± 0.5	1.9 ± 0.2
P-4000	1%	46.5 ± 3.3[a]	12.0 ± 1.1[a]	3.0 ± 0.3
	0.5%	42.3 ± 1.9[a]	12.1 ± 0.8[a]	2.7 ± 0.4
	0.25%	34.4 ± 2.7	9.3 ± 0.4	1.6 ± 0.4
	0.1%	34.3 ± 1.5	10.5 ± 0.8	1.9 ± 0.2
Dulcin	1%	58.3 ± 7.7[b]	11.7 ± 1.0[a]	9.3 ± 1.6[b]
	0.5%	39.9 ± 2.8[a]	9.3 ± 0.7[a]	5.0 ± 0.7[a]
	0.25%	33.7 ± 3.2	7.2 ± 0.6	4.8 ± 1.6
	0.1%	31.4 ± 1.5	8.7 ± 0.5	3.0 ± 0.6

[a] $p < 0.05$.
[b] $p < 0.01$.

1 mm. in diameter, poor in lymphoid cells and congested. The sinuses were not distended and contained moderate numbers of hemosiderin-laden macrophages. These local changes increased in severity with dosage and at the 1.0% dosage level several spleens had a markedly distorted structure because of the irregular distribution of hyperplasia, atrophy, and fibrosis.

In the kidney, pigmentation of the proximal convoluted tubular epithelium by a brown, chiefly nonferrous pigment which paralleled in degree and relation to dosage the hemosiderosis in the liver, was the only change attributable to dulcin. The relatively increased kidney weight (Table II) was not accompanied by an increased incidence of focal nephritis, fibrosis, etc., as will be mentioned in connection with P-4000. It should be remembered that the weight difference was smaller with dulcin, and that the dulcin animals showed greater growth retardation.

P-4000.—The chief gross and microscopic change caused by P-4000 was a pigmentation of the thyroid gland, seen at 0.25% and more in the diet. At 1.0% the thyroids were uniformly a dark reddish black in the fresh condition and almost coal black after formalin fixation. Slight enlargement and epithelial hyperplasia were also present. The pigment, in brown granules of about 1 μ diameter, filled the epithelial cells and to a lesser extent the follicular lumens. The appearance of the pigment, together with the results of various special staining reactions, eliminated P-4000 itself, hemofuscin, hemosiderin, ceroid, or carotenoid as the pigment, and narrowed the possibilities to either a wear-and-tear pigment or, perhaps more likely, a melanin. We have not seen such thyroid pigmentation before in any of our numerous chronic toxicity experiments. Our normal older rat thyroids may contain a very small amount of pigment, differing somewhat in appearance from that seen with P-4000; the quantitative difference between the two is of the order of 100:1 at the 1.0% level.

At 1.0%, some of the urinary bladders contained a soft yellowish gelatinous precipitate which dried into a light powder. Analysis showed the principal constituent of this powder to be protein, together with some uric acid and ammonium urate crystals. The yellow color came from P-4000, which likewise stained the urine, gastrointestinal tract, and fur of the animals.

The slight enlargements of the liver and spleen at the two highest dosage levels (Table II) were reflected microscopically in slight hepatic cell enlargement and, in the spleen, slight pulp hyperplasia and congestion, in some of the animals. Similarly, the increased kidney weights (and, grossly, increased pitting and fibrosis) correlated with an increased incidence of focal nephritis (nephrosis) at 0.1% or more of P-4000; the histological features of the nephritis were not changed. In tabulating the incidence of nephritis, the break came between 0.01% and 0.1%. Combining the two lowest dosage groups and the similarly appearing controls into one group gave 21% with marked nephritis and 36% with moderate or marked nephritis. Similarly combining the rats on 0.1% or more gave percentages of 31 and 59, respectively.

Saccharin.—No pathological effect whatever could be attributed to saccharin at levels of 1.0% or less. At 5% only one effect was noted, in the latter part of the experiment, namely an increased incidence of the ordinarily uncommon condition of abdominal lymphosarcoma. In the 5% group, there were seven animals with lymphosarcomas; this number is not out of line with the incidence in comparable groups of rats, but the fact that in four of the seven rats abdominal as well as thoracic lymphosarcomas were present is unusual, since ordinarily the ratio is about 1 to 15-20. Three of these four combinations occurred in animals on experiment one hundred and two or more weeks.

Cyclamate Sodium.—As with saccharin, the groups on dietary levels of 1% or less showed no changes which could be attributed to the cyclamate sodium. At the 5% level the only gross and microscopic effects were those of mild inanition. The animals at this dosage level had a moderate amount of diarrhea throughout the experimental period.

Hematology.—Blood studies were made once during the first year and twice during the second year of the experiment on 10 rats from each group of the higher dosage levels of each substance. The group on 1% dulcin showed a significant reduction in the erythrocyte count from an average of about 8 million in the controls to about 5 million in the experimentals and numerous normoblasts in the blood stream. The groups on 0.5% dulcin had a few normoblasts and a slightly, but not significantly, reduced average erythrocyte count. All other groups were normal.

DISCUSSION

The results of the above experiments show that the four synthetic sweetening agents may be divided into a relatively nontoxic group and a toxic group. Saccharin and cyclamate sodium belong to the former, and dulcin and P-4000 to the latter. The nontoxicity of saccharin has been well known both from extensive animal experimentation (3, 4) and from a long use. The present experiment on cyclamate sodium supports our earlier report (5) and the recent data of Richards, *et al.* (6). Richards, *et al.* (6), likewise found that the only toxic symptom of cyclamate sodium to animals was diarrhea at high levels. This also was observed clinically if persons took several grams of cyclamate sodium at one time. Although the sweetness equivalent of cyclamate sodium is about eight times less than saccharin, it appears to be safe for use as a saccharin substitute. There were no distinct toxic effects at levels as high as 5% in the diet of rats, and diarrhea seems to be the warning signal for the maximum tolerated dose both in animals and in man. It has been known that large doses of dulcin would produce toxic effects (7, 8); however, no previous experiment has shown the extent of such damage from small repeated dosages. From experiments in dogs lasting only twenty days, Kling, *et al.* (9), suggested the possibility of an accumulative effect of dulcin. Because of the extensive damage observed in our experiment, dulcin cannot be considered safe for food or drug use, even in small quantities. Lehmstedt (10), although mentioning the possibility of toxicity from compounds of the nature of P-4000, emphasized its high sweetening power. Since about a tenth as much P-4000 is required to give the sweetness equivalent to saccharin, this should be considered in a final analysis of its usefulness; however, in our experiments the ratio of toxicity is less than this ratio of effectiveness. P-4000 also has an undesirable anesthetic property. Thus, P-4000 cannot be considered to be a safe or desirable sweetening agent.

SUMMARY

1. In two-year feeding studies on rats, saccharin and cyclamate sodium were found to be without effect at levels of 1.0 per cent or less in the diet, and to cause only slight toxic effects at 5.0 per cent.

2. Dulcin (4-ethoxy-phenylurea) was toxic to rats at dosage levels of 0.1 per cent and upward. Adenomas of the liver up to 2.5 cm. in diameter occurred in half of the animals on 1.0 per cent, and in a few at lower levels down to 0.1 per cent of the diet. Splenic enlargement and darkening, associated with chronic congestion, pulp hyperplasia, and increased hemosiderin content, began at the 0.1 per cent level and increased parallel with dosage. Anemia and decreased growth rate were present at 1.0 and 0.5 per cent, as were increases in mortality rate, and in the number of circulating normoblasts.

3. P-4000 (1-*n*-propoxy-2-amino-4-nitrobenzene) was toxic to rats at dosage levels of 0.1 per cent and upward. An unusual pathological change was the presence of large amounts of a melanin-like pigment in the thyroid; at the 1.0 per cent level the thyroids were slightly enlarged and almost black. Pigmentation was pronounced at 0.5 per cent and slight but distinct at 0.25 per cent. A somewhat increased incidence of focal nephritis was present at 0.1 per cent and upward. Dosage levels of 1.0 and 0.5 per cent decreased growth rate and increased the relative size of the liver and kidney. The 1.0 per cent level increased mortality rate.

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Serum Levels After Oral Administration of Terramycin Base*,†,‡

By RACHEL MASON, PATRICIA KICE, ELDON L. CAFFERY, and M. M. MUSSELMAN

A comparison was made of the level of terramycin obtained in the serum of patients after single oral doses of terramycin base and terramycin hydrochloride. Serum levels were determined by a tube dilution method. The serum levels obtained after terramycin base were comparable to those obtained after terramycin hydrochloride. It is concluded that the two substances are absorbed equally well.

THERAPEUTICALLY effective levels of terramycin may be produced in the blood serum by oral administration of terramycin hydrochloride (1, 2). Terramycin hydrochloride is a salt of the amphoteric parent substance, terramycin base. In order to estimate the possible effectiveness of terramycin base as a therapeutic agent we have compared the levels of terramycin in the blood serum after single oral doses of the base and the hydrochloride.

METHOD

Serum levels of terramycin were determined by a tube dilution method in which the final total volume was 2 ml. (3, 4). Brain heart infusion broth (Difco) was the medium and diluent, and the test organism was a stock strain of an F. D. A. *Micrococcus pyogenes* var. *aureus*, which was employed in a dilution of 1:10,000 in an eighteen-hour broth culture. The stock standard of the drug was a solution of 200 μg. of crystalline terramycin hydrochloride in 1 ml. of broth. One hundred milligrams of the terramycin was weighed on an analytical balance and dissolved in 500 ml. of broth. The solution was then passed through a Seitz filter. This stock standard was prepared once a week and stored in the refrigerator at 4°. The working standard of terramycin, containing 1 μg./ml., was prepared daily from the stock standard by diluting it 1:200 with broth.

Terramycin was administered to patients orally at 8:00 a. m., and blood samples were drawn at 9:00 a. m., 10:00 a. m., 12:00 noon, 2:00 p. m., and at 8:00 a. m. the next day. The samples were refrigerated until all were collected. The storage period did not affect the concentration of terramycin in the serum.

Two parallel series of constituents were set up in tubes as shown in Table I. Both series of tubes were incubated at 37° overnight. The tube of highest dilution showing complete inhibition indicated the end point. The strain of *M. pyogenes* var. *aureus* was usually inhibited by 0.15 to 0.25 μg. of terramycin hydrochloride per milliliter of broth. The serum concentration of terramycin, in μg./ml., was obtained by multiplying the highest dilution of serum showing no growth by the smallest concentration per milliliter of terramycin hydrochloride showing no growth. The range of the concentration of terramycin which could be measured in serum by this procedure was from 0.3 to 12.0 μg./ml.

Tests were conducted on a total of 36 patients, consisting of three groups of 12. The patients in group I received 1.0 Gm. of terramycin base orally in a single dose; those in group II, 2.0 Gm.; and those in group III, 3.0 Gm. Serum levels of terramycin were determined at one, two, four, six, and twenty-four hours after administration of the drug. For control, parallel tests were made using terramycin hydrochloride, giving similar doses to the same patients.

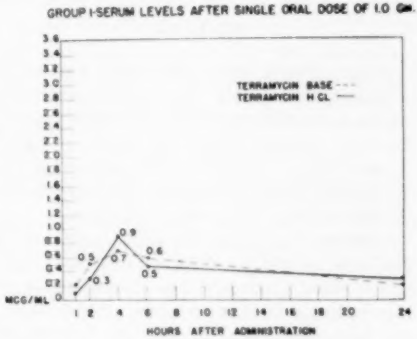

Figure 1

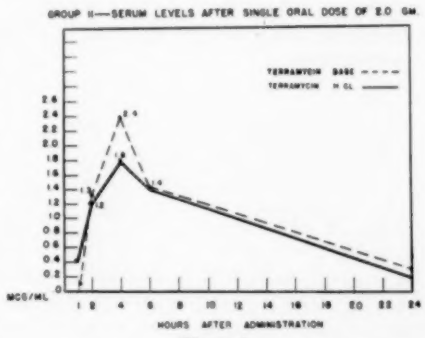

Figure 2

* Received July 9, 1951, from the Departments of Pathology, Urology, and Surgery, Wayne County General Hospital, Eloise, Mich.

† Presented at Regional Meeting of the American Federation for Clinical Research, Detroit, Mich., March 2, 1951.

‡ This investigation has been supported in part by Chas. Pfizer and Co., Inc., Brooklyn, N. Y.

TABLE I.—TUBE DILUTION METHOD FOR DETERMINING SERUM CONCENTRATIONS OF TERRAMYCIN

	Terramycin Standard									
Tube No. →	1	2	3	4	5	6	7	8	9	10
Terramycin in broth (1 μg./ml.), ml.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Broth, ml.	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1:10,000 dilution *M. aureus*, ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Final concentration of terramycin (μg./ml.)	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05

	Serum Sample										
Tube No. →	1	2	3	4	5	6	7	8	9	10	11
Serum, ml.	1.0	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05
Broth, ml.	0	0.5	0.55	0.6	0.65	0.7	0.75	0.8	0.85	0.9	0.95
1:10,000 dilution *M. aureus*, ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Final concentration of serum, ml.	0.5	0.25	0.225	0.2	0.175	0.15	0.125	0.1	0.075	0.05	0.025
Final dilution of serum	1:2	1:4	1:4.4	1:5	1:5.7	1:6.7	1:8	1:10	1:13.3	1:20	1:40

In order to evaluate the possible influence of age on absorption of terramycin, each group of 12 subjects was composed of four patients between the ages of 18 and 40 years, four between the ages of 40 and 60 years, and four over 60 years of age. In order to evaluate the possible influence of gastric acidity on absorption, the gastric juice of each patient was analyzed for free hydrochloric acid and *p*H value in the fasting state and for free hydrochloric acid after stimulation by histamine, before administration of the base and before administration of the hydrochloride.

RESULTS

Some patients had higher serum levels after administration of the base while others had higher levels after administration of the hydrochloride. But, as shown graphically in Figs. 1, 2, and 3, there was no marked difference between the average serum levels after terramycin hydrochloride and those after terramycin base.

Statistical analysis of the tabulated figures by Professor Donald Mainland of New York University revealed no suggestion of a significant difference in serum level at six hours. In the case of the four-hour levels, he felt the considerable variation in the results made the difference not significant. These determinations indicate that terramycin base is absorbed as well as terramycin hydrochloride.

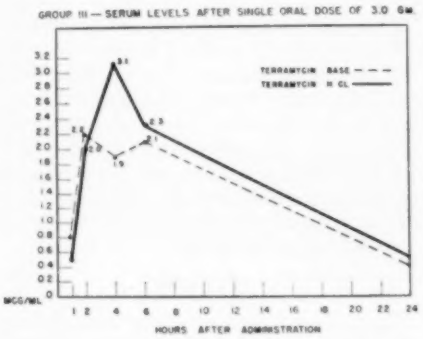
GROUP III — SERUM LEVELS AFTER SINGLE ORAL DOSE OF 3.0 Gm.
TERRAMYCIN BASE
TERRAMYCIN H. CL
MCG/ML
HOURS AFTER ADMINISTRATION

Figure 3

The data accumulated were not significant in regard to the relation of variations in serum levels to differences in ages of patients or gastric acidity

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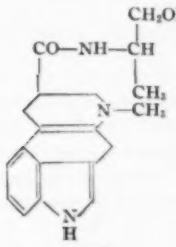
Indole Derivatives Related to Lysergic Acid*,†

By FRANK C. WHEELER,‡ GLENN L. JENKINS, and GUSTAV E. CWALINA

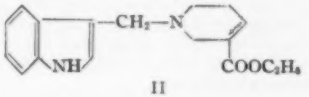
Several new compounds related to lysergic acid have been prepared and characterized. Four of these compounds are indole derivatives which have been prepared in quantities sufficient for pharmacological testing. Methods for the successful synthesis of the following are described: ethyl ester of 1-(3-indolylmethyl)guvacine, 3-indolylmethyl-di(β-carbethoxy-ethyl)amine, 1-(3-indolylmethyl)guvacoline, and ethyl 1-(3-indolylmethyl)nipecotate.

THE older preparations of ergot which contain all of the alkaloids have been quite extensively replaced in modern medicine by pure ergonovine, the alkaloid which stimulates the uterine musculature without the undesirable side reactions of the other ergot alkaloids (1). The relatively recent discovery of this alkaloid (2) has stimulated considerable chemical investigation and its formula has been rather definitely established by the extensive researches of Jacobs, Craig, *et al.* (3), in this country and by Smith and Timmis (4) in England. These workers found the basic structure essential to the pharmacodynamic properties of ergonovine to be lysergic acid. Although lysergic acid has not been synthesized, a number of derived compounds, which compare favorably in physiological activity with ergonovine, have been partially synthesized using the less desirable ergot alkaloids as a source of the acid (5). A rather critical shortage of ergot exists at times, due to inadequate cultivation of the crude drug. The synthesis of a simpler compound with the desirable oxytocic activity of ergonovine presents a challenging problem of considerable interest.

A sufficient number of compounds which are similar to ergonovine (I) in structure have not been prepared and tested to determine which portions of the molecule are essential for its activity. In the present investigation the indole nucleus was considered as a possible contributing feature to activity and attempts were made to prepare a group of indole derivatives which would also embrace some of the other chemical groups which distinguish ergonovine. This goal was partially realized in the preparation of the ethyl ester of 1-(3-indolylmethyl)guvacine (II). Structures which are common to both this compound and to ergonovine include a nitrogen heterocycle, which contains a double bond, attached to an indole nucleus. Furthermore, the alkanolamide group in one and the ester group in the other are both in *beta*-position to a tertiary amino group. Apparently the double bond is essential to the activity of ergonovine, for the dihydro derivative does not manifest an oxytocic effect. However, the double bond in II, like that in the isolysergic acid derivatives, in which it is contained in 9–10 rather than in 5–10 position, may be adversely located in the molecule. It seemed desirable to substitute an alkanolamide group for the ethoxy group in the ethyl ester of 1-(3-indolylmethyl)guvacine in order to determine whether the amide linkage might impart the desired activity. This proved to be much more difficult than was anticipated and could not be accomplished by any of the several methods tried. The method by which Stoll and Hofmann (5) con-



I



II

verted the ergot alkaloids to alkanolamides consists of the conversion of the alkaloids to lysergic acid hydrazide, thence to the acid azide by the action of nitrous acid, and finally the condensa-

* Received May 5, 1950, from the Research Laboratories, Purdue University, School of Pharmacy.
† Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.
‡ Fellow of the American Foundation for Pharmaceutical Education, 1947–1949. Present address: Eli Lilly and Co., Indianapolis, Ind.

tion of the acid azide with the appropriate amine. Attempts to prepare substituted amides of 1-(3-indolylmethyl)guvacine by this series of reactions were unsuccessful, probably because a pure hydrazide could not be isolated when the ester was treated with hydrazine (6). When the ester was subjected to the action of diethylamine at high temperature (185°) and high pressure (200 atmospheres) in an attempt to prepare the diethylamide directly, it was decomposed. At 160–165° the original ester was recovered. Likewise, attempts to prepare this compound by condensation of guvacine diethylamide with indole and formaldehyde in a manner analogous to that for the preparation of the ethyl ester (II) failed. When the ethyl ester of 1-(3-indolylmethyl)guvacine was refluxed with ethanolamine, with 2-amino-2-methyl-1-propanol, or with *n*-butylamine, the expected substituted amides could not be recovered from the resinous residues. When the dihydro derivative of II, ethyl 1-(3-indolylmethyl)nipecotate, was refluxed with various amines, similar failures resulted. In each case either the original ester was recovered or the reaction produced a dark, resinous material from which no substituted amide could be isolated. Baltzly, *et al.* (7), also experienced considerable difficulty in the preparation of the ethanolamides of certain phenethyl-β-alanine derivatives when the esters were refluxed with ethanolamine.

EXPERIMENTAL

Preparation of the Ethyl Ester of 1-(3-Indolylmethyl)guvacine.—The method of Snyder, *et al.* (8), was slightly modified in this synthesis. A solution of 2.91 Gm. (0.019 mole) of guvacine ethyl ester (9), prepared from guvacine hydrochloride (10), in 2.63 Gm. (0.044 mole) of glacial acetic acid, and 1.52 Gm. (0.019 mole) of 37% solution of formaldehyde, both solutions having been cooled to 5°, were added dropwise to 2.20 Gm. (0.019 mole) of indole. The mixture was agitated to effect solution and it was allowed to stand at room temperature for sixteen hours. Ten milliliters of water and 25 ml. of ether were added and the mixture was cooled to 0° and made alkaline with cold 15% solution of sodium hydroxide. It was extracted with the ether and the ether layer was decanted. Extraction with ether was repeated twice more. Upon evaporation of the combined ether extracts, a thick, light amber, viscous oil remained which was dried in a vacuum and crystallized by the addition of anhydrous ether. When crystallized three times from hot benzene, there remained 3.85 Gm. (77.1%) of white needles which melted at 98 to 98.5°.

Anal.—Calcd. for $C_{17}H_{20}N_2O_2$: N, 9.85%. Found: N, 9.77%.

Synthesis of 3-Indolylmethyl-di(β-carbethoxyethyl)amine.—This compound, prepared from equimolecular quantities of indole, formaldehyde and di-(β-carbethoxyethyl)amine, in the manner described above, was recrystallized from ether-petroleum ether, and then from ether; m. p. 82°; yield 17.2%. The hydrochloride melted at 122°.

Anal.—Calcd. for $C_{19}H_{28}N_2O_4$: N, 8.08%. Found: N, 8.34%.

Synthesis of 1-(3-Indolylmethyl)guvacoline.—Equimolecular quantities of indole, formaldehyde, and guvacoline (guvacine methyl ester) (9) were reacted as described under the preparation of the ethyl ester of 1-(3-indolylmethyl)guvacine. The product, after recrystallization from ether-petroleum ether, melted at 158°.

Anal.—Calcd. for $C_{16}H_{18}N_2O_2$: N, 10.36%. Found: N, 10.38%.

Synthesis of Ethyl 1-(3-Indolylmethyl)nipecotate.—Equimolecular quantities of indole, formaldehyde, and ethyl nipecotate (11) were reacted using the procedure described above under the preparation of the ethyl ester of 1-(3-indolylmethyl)guvacine. The reaction yielded 87.8% of a white crystalline compound which melted at 87–89° with previous softening.

Anal.—Calcd. for $C_{17}H_{22}N_2O_2$: N, 9.78%. Found: N, 9.21%.

SUMMARY

1. Four new indole derivatives have been prepared which have certain structures which are also contained in lysergic acid. These compounds have been prepared in sufficient quantities for pharmacological testing.

2. Attempts to prepare substituted amides from these new indole derivatives by various methods were unsuccessful.

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The Essential Oil of *Bursera microphylla**

By C. E. BRADLEY and A. J. HAAGEN-SMIT

The essential oil of leaves and twigs of *Bursera microphylla* consists of 80 per cent terpenes, 9 per cent sesquiterpenes and oxygenated compounds, and 8 per cent gums and resins. The terpene fractions consist largely of *d*, α- and β-phellandrene, tetrahydrocuminic acid (phellandric acid), and sesquiterpenes.

THE BURSERACEA (after the German botanist, Joachim Burser, 1593–1689) are a family of trees or shrubs comprising over 300 species. The Bursera family are sources of aromatic gums and resins such as copal, dammar, and elemi of commerce, and have been used since earliest times by the ancients in preparation of perfumes, frankincense, and myrrh. Linaloewood, an important perfume oil, is obtained from various Bursera species native to Mexico, Brazil, and French Guiana.

Two of these species are native to Arizona and California, namely, *Bursera microphylla* and *Bursera odorata*. Asa Gray (1) discovered *B. microphylla* in 1861, describing it in the *Proceedings of the American Academy of Arts and Sciences*. *Bursera microphylla* is found in southwestern Arizona, southeastern California, and Mexico. It grows on rocky slopes up to 2,500 feet elevation. The plant is sensitive to frosts and is confined to relatively small areas of moderate elevation.

The material for the following studies was collected in the Fortuna Mountains near Yuma, Ariz., where the microphylla grows quite extensively. The trees vary in size from tiny seedlings to specimens 20 ft. in height and 15 in. diameter at the base. The leaves are small, varying from 2–8 mm. in length and 1–2 mm. in width. They are thinly set on the branches, giving the appearance of pin feathers on a fowl's wing and adding to the grotesqueness of the tree. The short and stubby limbs have earned for it the title of "elephant tree." The bark on the old trees varies from 0.75 to 1.25 in. in thickness. The water extract of the bark is a deep red, from a soluble coloring material it contains. This bark has been used for tanning and medicinal purposes in Mexico.

The ash content of the bark is 10 per cent. Some Bursera ash has been found to contain radioactive materials. *Bursera microphylla* bark ash was not radioactive. The freshly cut green bark frees a milky exudate which darkens and hardens on drying. This resinous material has been utilized for incense in Mexican churches; also as a cement.

The essential oil is located chiefly in the leaves and bark of the young twigs, especially at the base of the spurs to which the leaves are attached. When the leaves are plucked from a lush, well-watered tree, tiny jets of oil are ejected from the spur base to a distance of 1–2 in. from the source—showing the oil is under pressure in the plant. If the branch is removed from the tree, oil does not flow on removing the leaf spur.

When favorably situated for a good supply of water, *B. microphylla* bears heavy crops of fruit—as much as one-half gallon of the purplish berries to a single shrub. The berries are about the size of a currant, and contain a single, three-angled, orange-red seed. The ripe fruit is very oily to touch, and in distillation with steam yields 1 per cent of an oil similar to that obtained from the leaves. The dry fruit contains 5 per cent of a soft yellow wax, which melts at 57° and is soluble in ether.

EXPERIMENTAL

Collection and Distillation.—Three collections were made of leaves and young twigs, mostly in collection 2. In collecting, trees were selected which showed healthy growth and carried the maximum amount of green foliage. Collection 1 was shipped by rail some 300 miles to the point of distillation, which was carried out on the third day after collections. Collection 2 was distilled at the point of collection, only a few hours after the material had been gathered, thus avoiding losses by evaporation and chemical changes taking place in the oil by delayed distillation. Collection 3 was made when the plant was dormant.

Table I contains comparative data obtained from collections 1, 2, and 3, showing dates of collection and yield of oil on green and dry shrub basis.

It is evident from the data given in Table I that in collections 1 and 3 there had been considerable loss of light-fraction oil by the method of handling the material previous to distillation. Collection 2 furnished 125 Gm. of oil which was obtained as follows:

A ten-gallon tin-lined still equipped with a glass condenser was partly filled with water and charged with a mixture of the leaves and twigs. The green material does not pack, but allows good circulation of steam through the charge, making the oil recovery rapid and complete. The distillate is a colorless, mobile oil with a sharp terpene odor. On ex-

* Received July 22, 1951, from the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena 4, Calif.

posure to light the oil gradually thickens and turns yellow.

Fractionation.—The results obtained in fractionating the Bursera oil are shown in Table II. Fractions 1 and 2 are colorless oils with a pronounced phellandrene odor These fractions make up about 80% of the total oil content. Fractions 3, 4, and 5, totaling about 9% of the oil, and the residue, fraction 6, representing approximately 8%, have an increasing yellow shade and a pleasant sesquiterpene odor.

Carbon and hydrogen determinations (C, 87.99% and H, 12.08%) show fraction 1 to be a pure terpene, $C_{10}H_{16}$. This fraction has the characteristic odor of phellandrene. The boiling point (174° at 754 mm.), the density (d_4^{27} 0.8389) and the refractive index (n_D^{25} 1.4708), are in close agreement with those reported for α- and β-phellandrene (6).

Identification of Phellandrene in *B. microphylla* Oil.—By the method of Wallach (2) the nitrosite of fraction 1 was prepared. After recrystallization from chloroform and methyl alcohol, white needles were obtained, m. p. 109°. Melting points of phellandrene nitrosites, covering the different forms, have been reported from 96 to 120° (12). Additional evidence for the presence of phellandrene was found by oxidizing the first fraction to phellandral according to Wallach (9). The crystalline orange 2,4-dinitro-phenylhydrazone prepared from this melted at 204°, characteristic of phellandral.

Identification of β-Phellandrene.—According to the method of West (4), the nitrosochloride of β-phellandrene was prepared from fraction 1. The yield was 0.55 Gm., or 7% of the fraction. The nitrosochloride had a melting point of 108–109°. The specific rotation was +230°. Melting points of β-phellandrene nitrosochloride have been reported from 100 to 109° (5). West (4) found the specific rotation of β-phellandrene nitrosochloride to be +226.5°. The formation of a crystalline nitrosochloride proves the presence of β-phellandrene, as α-phellandrene forms a soluble nitrosochloride.

Identification of α-Phellandrene by the Preparation of the Maleic Anhydride Addition Product.—By the method of Goodway and West (7, 11) the maleic anhydride addition product of fraction 1 was prepared, m. p. 124°. The melting point of authentic maleic anhydride addition product of α-phellandrene is 124–125°. The presence of α-phellandrene in Bursera oil is thus confirmed, as β-phellandrene does not show this reaction under the above conditions. These tests show the presence of both α- and β-phellandrene in the oil of *B. microphylla*.

Identification of Phellandral.—Fractions 4 and 5 of the Bursera distillate give an orange-red precipitate with 2,4-dinitrophenylhydrazine which has a melting point of 203–204° characteristic of the aldehyde, phellandral (3). The density of fraction 5 is 0.9472^{25}, agreeing closely with the density of phellandral, 0.9487, as reported by Penfold (8).

Identification of the Crystalline Material Forming in Fractions 4 and 5 of Bursera Distillate.—On cooling fractions 4 and 5, needle-like crystals separated. These crystals are colorless, insoluble in water, soluble in hot alcohol, and can be sublimed without decomposition at their melting point, 143–144°. Similar crystals are formed when a portion of fraction 5, containing phellandral, is exposed to the air for several days after adding a few drops of 30% hydrogen peroxide. Tetrahydrocuminic acid (phellandric acid) is formed readily in the air by the oxidation of the aldehyde, phellandral. The melting point of the tetrahydrocuminic acid is recorded as 143–145° (10). The analysis of the crystals and the formation of identical crystals by oxidizing the phellandral-containing fractions with hydrogen peroxide show the presence of tetrahydrocuminic acid in the higher fractions of the oil of *B. microphylla*.

Oil from Bark and Wood of *B. microphylla*.—The wood of the *B. microphylla* yielded only traces of oil on distillation. Wounding the shrub, after the method used on Mexican linaloe, did not increase the oil content.

Several pounds of *B. microphylla* bark were collected February 16, 1951. Distillation of the green bark with steam gave a colorless oil; yield 0.2% on the green material, equivalent to 0.55% on the dry bark. The odor of the oil was of phellandrene with a slight aromatic background. The density of the bark oil was 0.8760^{25}, considerably higher than the leaf and twig oil from this tree. Analysis and fractionation showed the oil to consist largely of sesquiterpenes.

TABLE I.—ANALYTICAL DATA ON THE OIL OF B. MICROPHYLLA

Collection	1	2	3
Date	Feb. 23, 1949	March 30, 1949	Dec. 24, 1950
Oil on green shrub, %	0.50	0.88	0.90
Oil on dry shrub, %	0.60	1.76	1.50
Density 27/4	0.8521	0.8437	0.8515

TABLE II.—FRACTIONATION OF BURSERA OIL

Fraction	Pressure, Mm.	Boiling Point, °C.	Total Oil, %
1	20	70–72	59
2	2	40	21
3	2	70	3.5
4	2	90–95	4.1
5	2	95–100	1.2
6		Residue	8.2

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Book Reviews

Methods in Medical Research. Vol. 4. MAURICE B. VISSCHER, Editor-in-Chief. Year Book Publishers, Inc., Chicago, 1951. xiv + 306 pp. Illustrated. 14.5 x 22.3 cm. Price $7.

This is the fourth volume in a series of publications which present the methods utilized in medical research. The text represents the contributions of many experts, writing about their special fields of interest, and it includes many details in methodology that are omitted in journal publications of research.

The text material is divided into four sections under the following headings: I. Histochemical Staining Methods; II. Fluid and Electrolyte Distribution; III. Studies on Gastrointestinal Pressures, Innervation, and Secretions; and IV. Tissue Culture Methods. Each section has a short introduction by the respective Associate Editor. References are given after each author's contribution.

Of special interest is the comprehensive discussion of the development, construction, and analytical applications of the flame photometer. The apparatus is illustrated, and its scope, supervision, and limitations are discussed. The description of the procedure for the determination of minute amounts of sodium and potassium in biological materials by the flame photometer is a model of clarity. Clarity of presentation is uniformly good throughout the text.

The thorough treatment of the many complex procedures, involving expert knowledge in different scientific fields, makes this a very valuable reference work and research guide. It should be included in all research libraries and in the laboratories devoted to studies in medical research.

Experimental Spectroscopy. 2nd ed. By RALPH A. SAWYER. Prentice-Hall, Inc., New York, 1951. x + 358 pp. Illustrated. 15.5 x 23.8 cm. Price $6.65.

This book adequately serves the purpose for which the author states it was written. It presents with excellent clarity discussions on prism and grating spectrographs and the techniques of their use in research. The author, who is the Professor of Physics and Dean of the Horace H. Rackham School of Graduate Studies of the University of Michigan, has directed this work to the student at the graduate level and to the industrial research worker. The reader is referred to other publications for more elementary or more detailed coverage of specific subjects.

The second edition includes valuable information that was omitted from the first. The development and application of infrared spectroscopy has received careful attention. Also expanded are the treatments of the spectroscopy of the vacuum ultraviolet and spectrochemical analysis. Perhaps the next edition of this splendid book will include more information on the applications of the spectrophotometer to quantitative analysis in the wave lengths shorter than the infrared. This is not intended as criticism, but as a hopeful suggestion.

The writing, choice of type, style, and format merit the highest praise. However, there seems to be a general tendency in the publication of recent books to use paper that is so thin that the bulkiness of the product is reduced. This is accompanied with increased visibility of the print on the reverse page. This should not have happened with this splendid book in the field of optics.

This book is recommended to everyone who is interested in the study and use of physical and physicochemical procedures in research and practical applications.

Industrial Oil and Fat Products. 2nd ed. By ALTON E. BAILEY. Interscience Publishers, Inc., New York, 1951. xxiv + 967 pp. Illustrated. 16 x 23.5 cm. Price $15.

This book is intended as a text on oil and fat technology. It is an excellent text for its intended purpose and it is a very good general reference volume on oils and fats. It contains an extensive running bibliography for detailed references. The book is divided into four sections containing 23 chapters. The emphasis on the technological portion of the text is indicated by the fact that 17 chapters are devoted to the divisions on Industrial Utilization of Fats and Oils, and Unit Processes in Oil and Fat Technology. The other six chapters are devoted to the divisions on The Nature of Fats and Oils, and Raw Materials for Oil and Fat Products.

Data on individual oils and fats are condensed but include the latest available important information. The technological portions are developed in greater detail and they benefit from the author's experience in this field. This is particularly true of the chapters on plastic shortening agents and bakery products, and on the practical operations of refining, bleaching, deodorization, and hydrogenation of edible oils and fats. The theoretical consideration of catalysts, the preparation and application of different types of catalysts to hydrogenation of fats and oils are comprehensively and interestingly presented.

Although no special consideration is given to the uses of fats and oils in pharmaceuticals, much of the material is applicable to this field, especially the fine chapter on Soap and Other Surface-Active Agents. This book should be available wherever pharmaceutical research is carried on.

An Introduction to the Chemistry of the Silicones. 2nd ed. By EUGENE G. ROCHOW. John Wiley & Sons, Inc., New York, 1951. xiv + 213 pp. Illustrated. 15 x 23.5 cm. Price $5.

This book presents a survey of the present knowledge in the field of organic compounds of silicon, and it is intended for the use of chemists, engineers, and industrial designers. The first chapters describe in some detail the Simple Covalent Compounds of Silicon, The Carbon-Silicon Bond, Synthe-

sis of Organosilicon Compounds, and other fundamental considerations of the subject. The later chapters emphasize the silicone polymers which are commercially important and describe the methods of preparation, their chemical and physical properties, and their possible uses. The last chapter is devoted to tabulations of the physical properties of organosilicon compounds.

The author's experience in this field at the General Electric Company enables him to present the text material in a critical and interesting manner. He states that the similarity of the electronic structures of carbon and silicon has led to a general assumption of similarity in chemical behavior and resemblance between carbon-silicon and ordinary carbon-carbon bonds. This view allows the wholesale transfer of the principles of organic chemistry to the study of organosilicon compounds. The author disproves the assumption and then proceeds to utilize theoretical considerations and observed behavior to develop his thesis.

The running bibliography is alphabetically tabulated at the end of the book, which has an author index and a subject index.

There is no doubt that this book should be available to anyone who is interested in the chemistry of the silicones.

The Vitamin B Complex. By F. A. ROBINSON. John Wiley & Sons, Inc., New York, 1951. xi + 688 pp. 14.5 x 22.5 cm. Price $9.

This is a comprehensive treatise on the vitamin B complex and its known individual components.

The close relationsip of the members of the vitamin B complex, the similarity in their distribution in foodstuffs, their biological effects on animals, plants, insects, and microorganisms, and in their biochemical functions, is stressed throughout the book.

All aspects of the literature relating to the B complex have been selectively incorporated in this thorough review. The book is divided into 14 chapters, 12 of which are devoted to single factors of the complex. Another chapter is devoted to miscellaneous water-soluble growth factors. The first chapter is devoted to thiamine, and the same general plan is followed in presenting this and the following chapters. A short historical introduction is followed by the methods of isolation; then the methods of synthesis, giving the methods developed in different countries, are presented. The chemistry and physical properties are reviewed, and the natural occurrence and the physiological significance and applications are discussed. The author's comments and observations are interesting and are based upon his experience in the isolation and study of growth factors, their synthetic preparation, biological assay methods, and the synthesis of antagonists.

The author discusses the lack of absolute specificity of the vitamins of the B complex; there is generally a group of compounds, the activity of which increases to a maximum with one particular member. He points out the unusual fact that maximum vitamin B_1 activity is not exhibited by the natural compound, but by the next higher homolog.

A bibliography is given at the end of each subdivision in the chapters, and the extent of the reference coverage is indicated by the 33 pages of author index and 25 pages of subject index at the end of the book.

The author has compiled a great amount of valuable information which is presented in an interesting manner. This book should be included in all pharmaceutical reference libraries.

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BOOKS RECEIVED

The Drug Plants of Illinois. By LEO R. TEHON. Illinois Natural History Survey, Circular 44, Urbana, Ill., 1951. 135 pp. Illustrated. 15.3 x 23 cm.

An Introduction to Materia Medica and Pharmacology. 6th ed. By ELSIE E. KRUG and HUGH ALISTER MCGUIGAN. C. V. Mosby Company, St. Louis, 1951. 612 pp. Illustrated. 14 x 22.3 cm. Price $4.25.

Pharmaceutical Dispensing. 4th ed. By WILLIAM HUSA. Distributed by Husa Brothers, College and Dubuque Sts., Iowa City, Iowa, 1951. vii + 811 pp. Illustrated. 16 x 23.6 cm.

Selective Toxicity with Special Reference to Chemotherapy. By ADRIEN ALBERT. John Wiley and Sons, Inc., New York, 1951. xii + 228 pp. Illustrated. 11 x 17 cm. Price $1.75.

Hormones and Body Water. By ROBERT GAUNT and JAMES H. BIRNIE. Charles C Thomas, Springfield, Illinois, 1951. vii + 57 pp. 14.5 x 22.5 cm. Price $2.25.

A Doctor's Pilgrimage. By EDMUND A. BRASSET. J. B. Lippincott Company, Philadelphia, 1951. 256 pp. 14 x 21 cm. Price $3.50.

Preparation, Properties, and Technology of Fluorine and Organic Fluoro Compounds. CHARLES SLESSER, Editor, and STUART R. SCHRAM, Associate Editor. McGraw-Hill Book Co., Inc., New York, 1951. xxiii + 868 pp. Illustrated. 16 x 23.7 cm. Price $10.50.

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Approved Laboratory Technic. 5th ed. By JOHN A. KOLMER, EARLE H. SPAULDING, and HOWARD W. ROBINSON. Appleton-Century-Crofts, Inc., New York, 1951. xii + 1180 pp. Illustrated. 17.5 x 25.3 cm. Price $12.

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- ✓ It is packaged in convenient 225, 100, 50 and 25 pound fiber drums.

*Picks up moisture. Should be kept in tightly closed containers.

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Check every one of the seven Baker features. Determine for yourself how many of these features are important to you. Then ask for samples and prices.

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